MOLECULAR, ANTIGENIC AND BIOLOGICAL STUDIES OF LOUPING-ILL VIRUS VARIATION IN THE BRITISH ISLES

by

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TO

MY PARENTS

&

MY SIBLINGS;

FIONA, ANGUS AND DOUGLAS.

CONTENTS

ABSTRACT

Louping-ill (LI) virus is a member of the tick-borne encephalitis virus serocomplex in the genus *Flavivirus*. LI virus infection results in a biphasic encephalomyelitic disease which has been reported in many domestic animals and man. However, the vector of the virus, the sheep tick (*Ixodes ricinus*), is mainly associated with upland rough grazing pasture and therefore the disease most frequently affects sheep and wild red grouse (*Lagopus lagopus scoticus*). A vaccine, consisting of inactivated LI virus, has been available for sheep since the 1930s but the disease persists today. LI virus is the only flavivirus known to occur in the British Isles and exists at the end of a cline of tick-borne flaviviruses which has spread westward across Asia and Europe during the past 2,000 years. The spatial and temporal characteristics of LI virus evolution remain to be defined. Previous studies investigated the variation present among LI viruses in an attempt to understand the evolution of LI virus and further the knowledge of its epidemiology. The study presented here has extended this work with the investigation of antigenic, molecular and biological properties of 43 LI viruses collected from around the British Isles.

Antigenic analysis using monoclonal antibodies identified two types of naturally occurring escape variants. The amino acid substitutions responsible for the alternative phenotypes were identified and shown to reside within the envelope (E) protein at residues 308 and 311. Investigation of the biological properties of the LI viruses *in vitro* and *in vivo* illustrated differences among them, some of which can be associated with genetic determinants.

Molecular analysis of the viruses by determining the nucleotide and deduced amino acid sequence of the complete E gene or a representative fragment of the gene has enabled an extended investigation of genetic variation among LI viruses. There was a distinct correlation between genetic variation and geographic distribution, with clustering of viruses from particular areas implying the occurrence of microevolution within these regions. Grouping by geography is to be expected for tick-borne viruses with non-migratory hosts which are dispersed only sporadically into new geographic regions. The only exception to this is the Irish viruses which appear to represent two distinct virus populations existing in the same tick population.

Phylogenetic analysis of the sequence data implies that the ancestral LI virus was initially introduced into Ireland and at a later date into Great Britain via Wales. The virus was then transported to Scotland from where it was dispersed throughout Scotland, the north of England and Norway. More recently the virus was probably reintroduced into Ireland and also transported to the south-west of England. The nucleotide substitution rate was estimated for the LI viruses included in this study and used to calculate the dates when viral lineages diverged. This analysis implies that LI virus was introduced into the British Isles less than 600 years ago and that the most significant dispersal from Scotland occurred 100-200 years ago. The precise mode of LI virus distribution is not known, but the recent time-scale and the pattern of dispersal implicate the involvement of man and in particular the movement of livestock along specific transport routes.

DECLARATION

I hereby declare that this thesis has been composed by me and that the work has been carried out by me except where collaboration is gladly acknowledged.

This work has not been and is not currently being submitted for candidature for any other degree.

Kirsty McGuire

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ABBREVIATIONS

BDH	British Drug House
C	capsid
C57/BL	C57 black mice
cDNA	complementary DNA
CFA	cell fusing agent
CMC	carboxymethylcellulose
CNS	central nervous system
DEN	dengue
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
d_s	synonymous pairwise genetic distance
E	envelope
EDTA	ethylenediamine-tetra-acetate
ER	endoplasmic reticulum
FCS	foetal calf serum
FETBE	Far Eastern tick-borne encephalitis
g	gravity
GGE	Greek goat encephalitis
gm	gram
H&E	haematoxylin and eosin
HI	heamagglutination-inhibition
HIV	human immunodeficiency virus
IBDV	infectious bursal disease virus
ICTV	international committee on the taxonomy of viruses
IIF	indirect immunofluorescence
JE	Japanese encephalitis
kb	kilobase

kDa	kilo Daltons
KFD	Kyasanur Forest disease
KUN	kunjin
LGT	langat
LI	louping-ill
M	membrane
MAb	monoclonal antibody
MEA	meaban
mRNA	messenger RNA
MVE	Murray Valley encephalitis
NBCS	new born calf serum
NCR	noncoding region
NEG	Negishi
NS	nonstructural
OHF	Omsk haemorrhagic fever
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
p.f.u.	plaque forming units
p.i.	post inoculation
PK	cell line name for porcine kidney cells
POW	Powassan
prM	premembrane
PRN_{75}	reciprocal of the antibody dilution which reduced the plaque count by 75%
PS	cell line name for porcine stable kidney cells
RF	replicative form
RGF	representative gene fragment
RI	replicative intermediate form
RNA	ribonucleic acid
RR	Ross River

RT	reverse transcription
SIN	Sindbis
SLE	St. Louis encephalitis
SMB	suckling mouse brain
SRE	Saumarez Reef
SSE	Spanish sheep encephalitis
SW	Swiss white mice
Taq	*Thermus aquaticus*
TBE	tick-borne encephalitis
TSE	Turkish sheep encephalitis
TYU	tyuleniy
USB	United States Biochemical
VI	veterinary investigation
Vs	Vasilchenko strain of TBE
WN	West Nile
WTBE	Western European tick-borne encephalitis
YF	yellow fever

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CHAPTER ONE

Introduction

and

Literature Review

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1.1. Flavivirus Classification

The genus *Flavivirus* contains approximately seventy species of virus, the majority of which are transmitted by haematophagous arthropods, either mosquitoes or ticks. However, several flaviviruses have only been isolated from bats and small rodents. Many of the flaviviruses are of human and veterinary importance and result in fever, encephalitis and haemorrhagic fever. Flaviviruses of medical importance include; yellow fever (YF) virus, dengue (DEN) viruses, Japanese encephalitis (JE) virus; tick-borne encephalitis (TBE) virus, Kyasanur Forest disease (KFD) virus and St. Louis encephalitis (SLE) virus.

In 1927 the aetiological agent of yellow fever was the first flavivirus to be isolated (reviewed by Monath, 1990), while in 1932 louping-ill (LI) virus was the first tick-borne flavivirus to be isolated (Gordon *et al.*, 1932), and this virus will be discussed in detail later in the chapter. After these events other flaviviruses were isolated and shown by complement fixation, cross-neutralization and cross-protection studies to form an antigenically closely related group of viruses (Webster, 1938; Smithburn, 1942; Casals & Webster, 1944; Edward, 1950). With the advent of the haemagglutination-inhibition (HI) test broader levels of antigenic relatedness could be investigated and arthropod-borne viruses (termed arboviruses) were shown to fall into at least two groups; A and B (Casals & Brown, 1954; Casals, 1957). Group A and group B, subsequently reorganized as the genera *Alphavirus* and *Flavivirus* respectively, were classified in the family *Togaviridae* due to similarities in their mode of transmission and physicochemical characteristics, i.e. small, enveloped positive strand RNA viruses. However, Westaway *et al.* (1985) summarized characteristics of the structure, replication strategy and genomic organization of flaviviruses which suggested that the genus should be included in a new family, the *Flaviviridae*. This classification was accepted by the International Committee on the Taxonomy of Viruses (ICTV) and the genera *Pestivirus* and *Hepatitis C* were also included in the family *Flaviviridae* (Francki *et al.,* 1991). The name flavivirus originates from the Latin word *flavus*, which means yellow and refers to the prototype species of flavivirus, YF virus.

Serological studies identified antigenic clustering among the flaviviruses (Casals, 1963; Clarke, 1964) and the genus, at the time consisting of 42 viruses, was divided into seven serocomplexes on the basis of cross-neutralization tests using polyclonal hyperimmune antisera (Madrid & Porterfield, 1974). Six viruses, including YF virus, were left unassigned. Between 1974 and 1988 a further 24 species of flavivirus were identified; for example, Meaban (MEA) virus (Chastel *et al.,* 1985; Monath, 1990). A further cross-neutralization study incorporated the new viruses and identified eight serocomplexes leaving 17 viruses unassigned, as shown in Table 1.1 (Calisher *et al.,* 1989). An interesting feature of this classification scheme is that the serocomplex organization is supported by the known epidemiology and epizootiology of these viruses with respect to host associations and geographic location. Virus groups 1 and 4 are associated with ticks, while viruses of groups 3, 5, 6, and 7 are transmitted by mosquitoes. Virus groups 2 and 8 have been isolated only from vertebrate hosts, namely bats and rodents respectively, and no vector has been established. Group 1 viruses occur in Europe and Asia, viruses of group 2 and 6 occur in Africa and group 3 viruses have a world-wide distribution. The viruses of serocomplex 4 are associated with sea coasts while group 8 viruses occur in North America.

Oligonucleotide fingerprinting (Trent *et al.,* 1981, 1989; Ceolen & MacKenzie, 1988; Flynn *et al.*, 1989), RNA-DNA hybridization (Blok *et al.,* 1984: Kerschner *et al.,* 1986; Shamanin *et al.,* 1990), restriction and peptide mapping (Guirakhoo *et al.,* 1987, 1991b; Walker *et al.,* 1988), monoclonal antibody (MAb) analysis (Stephenson *et al.,* 1984; Monath *et al.,* 1986; Gould *et al.,* 1990) and sequence analysis (Mandl *et al.,* 1989b; Blok *et al.,* 1991, 1992; Marin *et al.,* 1995b; Poidinger *et al.,* 1996) are among the techniques that have been employed to investigate the flavivirus temporal, spatial and structural interrelationships. The conclusions of these studies largely support the classification of the flaviviruses based on antigenic criteria.

Since 1989 several new flaviviruses have been identified; for example, Iguape virus from Brazil was recognized as a distinct flavivirus in 1993 (Coimbra *et al.*, 1993). In addition, a new tick-borne flavivirus was recently isolated from *Ixodes dammini* ticks in North America. This virus may represent a distinct species of flavivirus or a subtype of Powassan (POW) virus (Telford *et al.*, 1997).

1.2. Flavivirus Structure and Molecular Organization.

The flavivirus virion consists of an icosahedrally symmetrical nucleocapsid 25-30 nm in diameter which is composed of an RNA genome and capsid (C) protein. This structure is surrounded by a lipid bilayer derived from host membranes and contains the small viral membrane (M) protein and the larger viral envelope (E) protein. The resulting virion is approximately 40-50 nm in diameter (Westaway *et al.*, 1985).

Complete and partial genome sequences have been determined for many flaviviruses (Rice *et al.*, 1985; Castle *et al.*, 1985, 1986; Wengler *et al.*, 1985; Deubel *et al.*, 1986, 1988; Dalgarno *et al.*, 1986; Zhao *et al.*, 1986; Mackow *et al.*, 1987; Mason *et al.*, 1987b; Sumiyoshi *et al.*, 1987; Trent *et al.*, 1987; Coia *et al.*, 1988; Hahn *et al.*, 1988; Mandl *et al.*, 1988, 1989b, 1991a, 1993; Lee *et al.*, 1990; Osatomi & Sumiyoshi, 1990; Pletnev *et al.*, 1990; Shiu *et al.*, 1991, 1992a; Cammisa-Parks *et al.*, 1992; Fu *et al.*, 1992; Venugopal *et al.*, 1992, 1994a, b; Gao *et al.*, 1993a, b; Gritsun *et al.*, 1993a, b; Marin *et al.*, 1995a). These analyses have identified a single type of flavivirus genome organization which consists of a single stranded, positive sense RNA molecule approximately 10-10.5 kilobases (kb) in length. The genome contains a single open reading frame (ORF) which encompasses approximately 95% of the RNA molecule and is flanked by 5' and 3'- noncoding regions (NCRs).

The first genome to be completely sequenced was that of the 17D vaccine strain of YF virus (Rice *et al.*, 1985). The 5'-NCR of this virus consists of 118 nucleotides followed by an AUG start codon. The ORF consists of 10,233 nucleotides which encode a polypeptide of 3,411 amino acid residues. The 3'-NCR is 511 nucleotides in length. The size of these three regions varies between species of flavivirus, the ORFs of POW and DEN type-4 viruses are 10,302 and 10,158 nucleotides in length respectively (Zhao *et al.*, 1986; Mandl *et al.*, 1993). The length of the 3'-NCR varies from 350 to 650 nucleotides in strains of TBE virus (Wallner *et al.*, 1995).

Regardless of the variation in the length of flavivirus genomes the basic organization remains the same. The ORF encodes ten proteins; the structural capsid (C), premembrane (prM) and envelope (E) proteins and seven nonstructural (NS) proteins. The proteins are encoded in the order 5'-C-prM-E-NS1-NS2a-NS2b-NS3-

NS4a-NS4b-NS5-3' (Chambers *et al.*, 1990a). The characteristics of each of these proteins are described below.

1.3. The Structural Proteins

1.3.1. The Capsid (C) Protein

The C protein has a molecular weight of between 13-16 kilo-Daltons (kDa) and on the basis of comparative sequence data is the least conserved of the flavivirus structural proteins (Deubel *et al.,* 1986; Mason *et al.*, 1987b; Sumiyoshi *et al.,* 1987; Pletnev *et al.,* 1990). The sequence for this protein begins one residue after the AUG start codon and the resulting initial methionine residue is presumably removed by cellular aminopeptidase during post-translational processing (Chambers *et al.,* 1990a).

Although the primary sequence of the C gene is not conserved there is a high percentage of basic residues in all flavivirus C proteins. Lysine and arginine residues make up approximately 25% of the protein (Rice *et al.,* 1985; Sumiyoshi *et al.,* 1987; Pletnev *et al.,* 1990). The C protein forms the nucleocapsid structure with the positively charged RNA genome and the basic amino acids of the C protein are believed to be important in neutralizing this positive charge to allow the formation of the nucleocapsid structure (Rice *et al.,* 1985). The N- and C- terminal domains of the mature C protein are important for specific binding with the NCRs of the flavivirus genome (Khromykh & Westaway, 1996).

The hydrophobicity plots are similar for all flavivirus C proteins (Deubel *et al.*, 1986; Mandl *et al.*, 1988) and contain two conserved hydrophobic stretches, the first between residues 31-54 and the second spanning the C-terminal 22 residues of JE virus (Sumiyoshi *et al.,* 1987). Proteinase K digestion during translation only affects the N-terminal 20-30 residues of the C protein, implying that the protein is closely associated to the endoplasmic reticulum (ER) and both hydrophobic domains have been implicated in this association (Ruiz-Linares *et al.,* 1989). Immediately

upstream of the terminal hydrophobic stretch is a potential cleavage site for the NS2b-NS3 protease which is conserved in all flaviviruses (Nowak *et al.,* 1989).

During the replication of WN virus two forms of the C protein are produced, termed anchored-C and virion-C. Anchored-C consists of the amino acid sequence up to the N-terminus of the prM protein, while the mature virion-C lacks the C-terminal hydrophobic stretch (Nowak *et al.,* 1989). The occurrence of multiple forms of the C protein has been reported in several different flaviviruses (Mandl *et al.,* 1988; Ruiz-Linares *et al.,* 1989; Lobigs, 1993; Amberg *et al.,* 1994; Yamshchikov & Compans, 1994). The C-terminus of the virion-C protein terminates with the dibasic residues of the putative NS2b-NS3 protease cleavage site (Speight & Westaway, 1989). Mutations at this cleavage site; the absence of the NS2b and NS3 proteins; or mutations in the catalytic triad of the NS3 protein result in the absence of the virion-C form of the C protein (Lobigs, 1993; Yamshchikov & Compans, 1994).

Several authors attempting to produce recombinant flavivirus vaccines have observed that the presence of the complete C protein resulted in poor immunogenic antigens due to the lack of prM protein processing and secretion of the prM and E proteins (Zhao *et al.,* 1987; Zhang *et al.,* 1988; Bray *et al.,* 1989; Konishi *et al.,* 1991). However, a good immune response was elicited by recombinants containing only the C-terminal hydrophobic stretch of the C protein (Konishi *et al.,* 1991; Qu *et al.,* 1993). In addition, co-infection of recombinants containing the entire C protein and those containing the NS2b and NS3 proteins resulted in prM protein processing and secretion of the prM and E proteins (Sato *et al.,* 1993). These results imply that the NS2b-NS3 protease complex has an important role in the processing of the structural proteins and that cleavage of anchored-C to virion-C precedes and is a prerequisite of cleavage at the C/prM junction (Ruiz-Linares *et al.,* 1989; Lobigs, 1993; Amberg *et al.,* 1994; Stocks & Lobigs, 1995).

Details of the interaction between the C, prM, NS2b and NS3 proteins are not fully understood and several models have been described (Nowak *et al.,* 1989; Lobigs, 1993; Yamshchikov & Compans, 1993). Lobigs (1993) suggested that after translocation of the prM protein, cleavage of anchored-C to virion-C causes a conformational change in the remaining hydrophobic stretch of the C and prM

proteins. This results in the C/prM junction becoming more accessible to signal peptidase. Yamshchikov & Compans (1993, 1995) hypothesized that the NS2b and NS3 proteins have a dual action in the processing of the C and prM proteins. Firstly the NS2b-NS3 protease complex cleaves anchored-C and secondly the NS2b protein interacts with the C protein to produce a conformational change which increases the accessibility of the signal peptidase cleavage site. However, digestion of the C protein by trypsin, in the absence of the NS2b and NS3 proteins, results in the production and secretion of the prM protein (Stocks & Lobigs, 1995). This implies that the NS2b-NS3 protease complex has a single function and by cleaving anchored-C a conformational change is elicited in the remaining polypeptide (Lobigs, 1993).

1.3.2. The Premembrane (prM) and Membrane (M) Proteins

The M protein is only present in mature extracellular virions while a larger protein is found in immature virus particles (Shapiro *et al.,* 1972). Shapiro and co-authors (1972) proposed that the larger protein, prM, was the precursor of the M protein and this was supported by sequence data (Castle *et al.,* 1985; Rice *et al.,* 1985; Wengler *et al.,* 1985). The C-terminus of the anchored-C immediately precedes the N-terminus of the prM precursor protein (Wright *et al.,* 1989). The prM precursor is glycosylated with a molecular weight of 18 kDa while the mature, non-glycosylated M protein has a molecular weight of 8 kDa and contains 75 residues (Sumiyoshi *et al.,* 1987; Coia *et al.,* 1988).

The prM protein contains six cysteine residues which are conserved among flaviviruses and form three intramolecular disulphide bonds within the pr portion of the prM protein, i.e. they are not present in the mature M protein (Nowak & Wengler, 1987; Trent *et al.,* 1987). The prM proteins of all flaviviruses investigated to date are glycosylated, although the number and location of potential sites vary (Rice *et al.,* 1985; Mason *et al.,* 1987b; Sumiyoshi *et al.,* 1987; Trent *et al.,* 1987; Coia *et al.,* 1988; Mandl *et al.,* 1988; Pletnev *et al.,* 1990). YF virus has three potential glycosylation sites, while DEN, JE, kunjin (KUN), SLE and TBE viruses have single

potential glycosylation sites which exhibit serocomplex-specific localization (Mandl *et al.,* 1988).

Flavivirus prM proteins have similar hydrophobicity plots (Trent *et al.,* 1987). In particular two hydrophobic domains at the C-terminus of prM and M proteins are conserved and separated by a single arginine residue (Ruiz-Linares *et al.,* 1989; Markoff *et al.,* 1994). The first hydrophobic domain acts as a stop-transfer signal and the second domain initiates translocation of the E protein (Depres *et al.*, 1990). In addition, both hydrophobic domains are required for stable insertion of the M protein into the lipid bilayer and efficient cleavage at the prM/E junction (Markoff *et al.,* 1994).

The M protein is partially exposed on the virion surface and MAbs raised against the M protein of DEN virus are capable of passively protecting mice (Kaufman *et al.,* 1989). In addition DEN type-4 virus prM and M proteins expressed in vaccinia elicit a protective immune response (Bray & Lai, 1991).

Immature virions, which contain the prM protein, have a lower infectivity than mature, M protein containing virions (Shapiro *et al.,* 1972; Wengler & Wengler, 1989b; Randolph *et al.,* 1990; Guirakhoo *et al.*, 1991a; Pletnev *et al.,* 1993; Heinz *et al.,* 1994b). This phenomenon is due to the association which exists between the M protein and the fusogenic E protein (Randolph & Stollar, 1990). Cell entry by flaviviruses is believed to involve receptor-mediated endocytosis and release of virions from endosomes (Gollins & Porterfield, 1985; Ng & Lau, 1988; Randolph & Stollar, 1990). The mildly acidic conditions in the endosome induce a conformational change in the E protein thus exposing a fusion peptide (Guirakhoo *et al.,* 1989; Allison *et al.,* 1995). During virus maturation the virion is located in mildly acidic post-Golgi vesicles, in conditions suitable for the E protein conformational change. In immature virions the prM protein forms heterodimers with the E protein (Wengler & Wengler, 1989b; Heinz *et al.,* 1994b) thus preventing the acid-mediated conformational change of the E protein and thereby preventing inactivation of the virus (Randolph *et al.,* 1990; Guirakhoo *et al.*, 1991a, 1992; Heinz *et al.,* 1994b; Allison *et al.,* 1995). The cleavage of prM to M, which occurs very late in the maturation process, results in a conformational change in the E protein

thus allowing the formation of homodimers (Guirakhoo *et al.,* 1992). This oligomeric rearrangement induces the infectivity of the virion.

In addition, the prM protein has been implicated as playing an important role in the correct folding and assembly of the E protein (Konishi & Mason, 1993). Baculovirus and vaccinia expression systems only induce protective immunity if the E protein is expressed in association with the prM protein (Brandt, 1988; Mason *et al.,* 1989, 1991; Konishi *et al.,* 1991, 1992; Pincus *et al.,* 1992; Shiu *et al.,* 1992b; Venugopal & Gould, 1994; Venugopal *et al.,* 1994c).

1.3.3. The Envelope (E) Protein

The E protein is the major structural protein of the flavivirus virion, with a molecular weight of 51-60 kDa. The E protein is the most conserved of the three structural proteins (Mandl *et al.*, 1989a; Venugopal *et al.,* 1994a) and there is complete conservation of the 12 cysteine residues which form six intramolecular disulphide bonds (Nowak & Wengler, 1987). However, there is considerable heterogeneity in the number and location of potential and utilized glycosylation sites within and between flavivirus species. Murray Valley encephalitis (MVE) virus has a single glycosylation site which is utilized (Dalgarno *et al.,* 1986; Winkler *et al.,* 1987a). Members of the TBE virus serocomplex encode three potential glycosylation sites within the E protein, only one of which is utilized, at residue Asn-154 in LI virus (Winkler *et al.,* 1987a; Shiu *et al.,* 1991). WN and KUN viruses have no potential glycosylation sites (Wengler *et al.,* 1985; Wright, 1982), while YF and SLE viruses exhibit strain variation and produce glycosylated and unglycosylated E proteins (Schlesinger *et al.,* 1983; Deubel *et al.,* 1987; Cane & Gould, 1989; Ballinger-Crabtree & Miller, 1990; Vorndam *et al.,* 1993).

The functional importance of the oligosaccharide is not known and mutagenesis at the glycosylation site or deglycosylation result in a range of effects, from increasing to decreasing the neurovirulence of the virus (Winkler *et al.,* 1987a; Kawano *et al.,* 1993; Pletnev *et al.,* 1993; Vorndam *et al.,* 1993).

The E protein is involved in several important functions including receptor-mediated endocytosis and acid-induced virus-endosomal membrane fusion which releases the viral RNA into the cytoplasm. The E protein also acts as the viral haemagglutinin and induces the protective immune response. E protein-specific MAbs can passively protect mice from infection (Gould *et al.,* 1986; Gao *et al.,* 1994). Due to the fact that a major proportion of the humoral response is directed against the E protein, the investigation of antigenic determinants has concentrated on this protein and type, serocomplex and flavivirus group reactive epitopes have been identified (Clarke, 1964; Trent, 1977). The use of MAbs has extended this analysis to identify: strain, type, sub-serocomplex, complex and flavivirus group specific epitopes (Dittmar *et al.,* 1980; Gentry *et al.,* 1982; Henchal *et al.,* 1982; Peiris *et al.,* 1982; Heinz *et al.,* 1983a; Kimura-Kuroda & Yasui, 1983; Schlesinger *et al.,* 1983; Stephenson *et al.,* 1984; Kobayashi *et al.,* 1984; Monath *et al.,* 1984; Gould *et al.,* 1985).

Three antigenic domains within the TBE virus E protein, termed A, B and C, were defined (Guirakhoo *et al.,* 1989) and used to construct a two dimensional structural model of the E protein (Mandl *et al.*, 1989a). The absolute conservation of the cysteine residues, the homology of the hydrophobicity profiles along with the fact that flaviviruses share similar regions of variable and conserved sequence suggests that the model is applicable to all flavivirus E proteins (Nowak & Wengler, 1987; Mandl *et al.*, 1989a; Gritsun *et al.,* 1995). Recently the three dimensional structure of a soluble form of the E protein of TBE virus was resolved and shown to exist as a homodimer which extends in a direction parallel with the viral membrane (Rey *et al.,* 1995). The monomer form of the E protein contains three domains; I, II and III, which correspond well with the antigenic domains C, A and B respectively.

Domain I contains residues 1-51, 137-189 and 285-302 (residues 145-170 in domain C) and forms the central domain of the E protein monomer. The domain contains variable sequence which encodes the majority of the sub-type specific epitopes (Heinz *et al.,* 1984; Guirakhoo *et al.,* 1989). The single glycan of TBE virus is attached to this domain and is important for its stabilization (Guirakhoo *et al.,* 1989). The disparity between the residues included in domains I and C is due to the

limited number of MAbs that were obtained in the earlier study and may reflect the relatively low immunodominant status of this domain in the E protein (Mandl *et al.*, 1989a).

Domain II is the dimerization domain and contains residues 52-136 and 190-284 (residues 50-125 and 200-250 in domain A). The sequence of domain II is the most conserved within the E protein (Venugopal *et al.*, 1994a) and contains the cross-reactive epitopes (Guirakhoo *et al.*, 1989). In particular, residues 98-111 are highly conserved and believed to form the fusion peptide (Roehrig *et al.*, 1989; Heinz *et al.*, 1990). This region is hydrophobic and contains the motif Gly-Leu-Phe-Gly which is also found in the HA2 fusion protein of influenza virus (Wharton *et al.*, 1988). In the three dimensional model residues 98-111 form a loop which is hidden by the other subunit of the dimer (Rey *et al.*, 1995).

In the immature virion the prM protein interacts with domain II of the E protein (Holzmann *et al.*, 1995) and MAbs which recognize epitopes within domain II block the acid-induced conformational change of the E protein (Kimura-Kuroda & Yasui, 1983; Vorovitch *et al.*, 1991). This conformational change, which leads to the oligomeric rearrangement of E proteins from homodimers to homotrimers (Allison *et al.*, 1995), only affects epitopes within domain II (Heinz *et al.*, 1983b; Guirakhoo *et al.*, 1989). The conformational change causes the exposure of hydrophobic elements which can result in the aggregation of virus particles and the fusion peptide is more accessible after exposure to acidic pH (Heinz *et al.*, 1994a; Roehrig *et al.*, 1990). The oligomeric rearrangement of the E protein is a two step process, with the disassociation of the dimers followed by reassociation of monomers to trimers. The rearrangement requires the C-terminal 20% of the E protein which includes the stem-loop portion of the protein (Stiasny *et al.*, 1996). Rey *et al.* (1995) proposed a model whereby low pH induces a conformational change at the base of domain II which acts as a hinge and elevates the tip of the domain thus exposing the fusion peptide loop to the cellular membrane.

Within domain II two short peptide sequences have been identified which can distinguish flaviviruses (Shiu *et al.*, 1991, 1992a). Serocomplex 1 flavivirus E genes encode the amino acid residues Glu-His-Leu-Pro-Thr-Ala (EHLPTA) at residues

207-212, which probably represents an insertion into the TBE serocomplex virus genome rather than a deletion in other flavivirus lineages because both mosquito-borne viruses and tick-borne members of the Tyuleniy (TYU) virus serocomplex, a monophyletic sister group of the TBE virus serocomplex, lack the hexapeptide code. This hexapeptide sequence is absolutely conserved in all TBE serocomplex viruses except POW virus and the newly identified virus from *Ixodes dammini*, which have the motif Asp-His-Leu-Pro-Ser-Ala (DHLPSA), and KFD virus, which has the motif Glu-His-Leu-Pro-Lys-Ala (EHLPKA) (Mandl *et al.,* 1993; Venugopal *et al.,* 1994a; Telford *et al.*, 1997). Therefore, although these three viruses have slightly different sequences, they still have a hexapeptide motif and there is no equivalent sequence present in other flaviviruses. The second genetic marker is a tripeptide at residues 232-234 which identifies individual species of flavivirus. TBE virus encodes the motif Ala-Gln-Asn (AQN) while LI virus encodes the genetic marker Asn-Pro-His (NPH). The functional importance of such conserved and variable domains is not clear although both motifs appear to be at least partially exposed on the virion of TBE virus and are recognized by MAbs (Mandl *et al.*, 1989a).

Domain III is a continuous domain containing residues 303-395 (residues 301-395 in domain B) and encodes mainly complex specific epitopes (Guirakhoo *et al.,* 1989). The domain is independently folded and can be isolated by trypsin digestion (Heinz *et al.,* 1983b, 1984; Winkler *et al.,* 1987b). The disulphide bond between cysteine residues 11 and 12, at residues 317 and 333 in LI virus, is required to maintain the conformation of domain III and its antigenic reactivity (Winkler *et al.,* 1987b; Guirakhoo *et al.,* 1989; Mason *et al.,* 1989; Wengler & Wengler, 1989a; Lin *et al.,* 1994).

The E protein determines cell tropism (Anderson *et al.,* 1992; Chen *et al.,* 1996a, b) and domain III has been implicated as playing a major role in this function as the site of receptor binding (Heinz *et al.,* 1990; Holzmann *et al.*, 1990). A substitution of Lys to Gln at residue 303 of the E protein of YF virus vaccine strain 17D-204 is associated with a change in cell tropism and results in an encephalitic disease instead of the usual viscerotropic disease (Jennings *et al.,* 1994). Recently it has been shown that residues 281-423, which includes domain III, in the E protein of DEN virus are

required for receptor binding (Chen *et al.,* 1996b). The three dimensional model of the E protein of TBE virus identifies an immunoglobulin-like (IgC-like) module within domain III. A similar structure is often found in cell-adhesion proteins and this provides further evidence that the E protein domain III is the site of receptor binding (Rey *et al.,* 1995).

An additional genetic marker has been identified within domain III. A pentapeptide motif at residues 320-324 is a serocomplex specific genetic marker (Gao *et al.,* 1993a). TBE serocomplex viruses encode the residues Asp-Ser-Gly-His-Asp (DSGHD) while the motif Asp-Thr-Gly-His-Gly (DTGHG) identifies members of the JE virus serocomplex.

Sequence analyses comparing the genomes of low and high virulence flaviviruses have identified the E protein as playing an important role in the virulence of the virus (Hahn *et al.*, 1987a; Dunster *et al.,* 1990; Lobigs *et al.,* 1990; Nitayaphan *et al.,* 1990; Blok *et al.,* 1992; Post *et al.,* 1992; Halevy *et al.,* 1994; Labuda *et al.,* 1994; Ni & Barrett, 1994; Ni *et al.,* 1994, 1995b; McMinn *et al.,* 1995b; Sanchez & Ruiz, 1996; Schlesinger *et al.,* 1996; Wallner *et al.,* 1996; Ryman *et al.,* 1997). In addition, mutagenesis analysis and the generation of neutralization MAb escape mutants, which generally result from a single amino acid substitution, have extended this analysis (Lobigs *et al.,* 1987; Mandl *et al.*, 1989a; Holzmann *et al.,* 1990; Cecilia & Gould, 1991; Hasegawa *et al.,* 1992; Sil *et al.*, 1992; Jiang *et al.,* 1993; Kawano *et al.,* 1993; Pletnev *et al.,* 1993; Gao *et al.,* 1994; Lin *et al.,* 1994; McMinn *et al.,* 1995a, 1996; Sumiyoshi *et al.,* 1995; Hiramatsu *et al.,* 1996; Holzmann *et al.,* 1997). The majority of virulence characteristics are associated with three regions of the E protein; the distal face of domain III, the hinge-like base of domain II and the interface between domains I and III and the fusion peptide loop of the other subunit of the dimer (Rey *et al.,* 1995). Mutations on the distal face of domain III are believed to alter the ability of the IgC-like module to bind to the putative cellular receptor which frequently results in loss of neuroinvasiveness or a change in cell tropism (Jiang *et al.,* 1993; Jennings *et al.,* 1994). Mutations in the other two locations presumably affect the fusion activity of the E protein (McMinn *et al.,* 1996).

1.4. The Nonstructural Proteins

1.4.1 The NS1 & NS2a Proteins

The NS1 protein is unusual among nonstructural proteins because it is glycosylated. The NS1 protein of LI virus has a molecular weight of 40-46 kDa and comprises 352 amino acid residues (Venugopal *et al.*, 1994b). The NS1 protein appears to have a conserved structure among the flaviviruses with complete conservation of 12 cysteine residues and similar hydrophobicity profiles (Chambers *et al.*, 1990a; Pletnev *et al.*, 1990). However, the number of potential glycosylation sites varies between flavivirus species; KUN, TBE and LI viruses have three potential sites (Coia *et al.*, 1988; Jacobs *et al.*, 1992; Venugopal *et al.*, 1994b) while DEN, YF and JE viruses have two potential glycosylation sites (Smith & Wright, 1985; Chambers *et al.*, 1990b; Depres *et al.*, 1991; Flamand *et al.*, 1992). The glycosylation site at residue 208 of LI virus is conserved in all flaviviruses (Pletnev *et al.*, 1990; Venugopal *et al.*, 1994b). The function of the oligosaccharides remains unclear although ablation of the glycosylation site at residue 130 of YF and DEN type-4 viruses result in attenuation of the virus due to reduced RNA synthesis (Pletnev *et al.*, 1993; Muylaert *et al.*, 1996).

The functional form of the NS1 protein appears to be a homodimer which is sensitive to boiling and acidic conditions of less than pH 6.0 (Winkler *et al.*, 1988; Depres *et al.*, 1991). The type of bonding involved in dimerization has not been elucidated fully, although the cysteine residues and structure at the C-terminus of the NS1 protein are involved (Hori & Lai, 1990; Parrish *et al.*, 1991; Pryor & Wright, 1993). In addition, the absence of the glycosylation site at Asn-207 in DEN type-2 virus results in dimer instability and reduced levels of NS1 protein secretion (Pryor & Wright, 1994). Unlike the monomer, which is generally hydrophilic, the dimer is hydrophobic and membrane associated (Winkler *et al.*, 1989; Fan & Mason, 1990; Depres *et al.*, 1991; Flamand *et al.*, 1992; Leblois & Young, 1995). However, the NS1 protein lacks a membrane anchor hydrophobic sequence and therefore is not as

hydrophobic as typical membrane associated proteins (Speight *et al.,* 1988; Winkler *et al.*, 1989; Depres *et al.,* 1991).

After dimer formation the NS1 protein is transported to the Golgi apparatus where the oligosaccharide at residue 208 in DEN and YF viruses is converted from a high-mannose to a complex glycan before secretion (Post *et al.*, 1990; Depres *et al.,* 1991; Flamand *et al.,* 1992; Jacobs *et al.,* 1992; Pryor & Wright, 1994; Muylaert *et al.,* 1996). Only the dimer form of NS1 is secreted from infected cells (Flamand *et al.,* 1992; Pryor & Wright, 1993) and in the extracellular medium TBE virus NS1 protein exists as pentamers and hexamers (Crooks *et al.*, 1994).

The NS1 protein is found associated with cell membranes, including the plasma membrane, in the infected cell cytoplasm and in the extracellular environment (Stohlman *et al.,* 1975; Cardiff & Lund, 1976; Gould *et al.,* 1985; Westaway & Goodman, 1987; Cane & Gould, 1988). The NS1 protein was initially described as the soluble complement fixing antigen found in sera of convalescent dengue patients (Smith & Wright, 1985).

The NS1 protein has been investigated as a vaccine candidate because of the strong immune response it invokes and the significant degree of antigenic homogeneity which exists between the NS1 proteins of several flaviviruses (Stephenson *et al.*, 1984; Crooks *et al.*, 1994). MAbs raised against the NS1 protein are capable of passively protecting mice from YF and DEN virus infections (Schlesinger *et al.,* 1985; Gould *et al.,* 1986; Henchal *et al.,* 1988). Purified NS1 protein induces a protective immune response in monkeys and mice against YF and DEN type-2 virus infections (Schlesinger *et al.,* 1985, 1986, 1987) and NS1 proteins expressed in *Eschericha coli*, vaccinia, baculovirus and adenovirus expression systems are also protective against TBE, JE, DEN and YF viruses (Cane & Gould, 1988; Zhang *et al.,* 1988; Bray *et al.,* 1989; Falgout *et al.,* 1990; Putnak & Schlesinger, 1990; Konishi *et al.,* 1991; Jacobs *et al.,* 1992; Qu *et al.,* 1993). The mechanism of this protection is not fully understood although the involvement of complement-mediated cell lysis has frequently been described (Schlesinger *et al.,* 1985, 1990; Falgout *et al.,* 1990; Henchal *et al.,* 1988; Jacobs *et al.,* 1992; Qu *et al.*, 1993). The protection provided by immunization with the NS1 protein varies

considerably and the strongest protection has been observed against the more attenuated viruses (Gould *et al.,* 1986; Falgout *et al.,* 1990). In addition, immunization with DEN type-2 virus NS1 protein provides no cross-reactive protection against DEN type-1 virus (Schlesinger *et al.,* 1987).

The NS2a protein is small and contains hydrophobic domains (Rice *et al.,* 1985; Pletnev *et al.,* 1990). There is limited sequence similarity exhibited between the NS2a proteins of flaviviruses, although the position of the hydrophobic stretches is conserved suggesting that the protein is membrane associated (Falgout & Markoff, 1995). During polyprotein processing a NS1-NS2a precursor is initially produced. This precursor is not cleaved in several vaccinia and baculovirus recombinants which express the NS1 and partial NS2a proteins (Falgout *et al.,* 1989, 1990; Parrish *et al.,* 1991; Falgout & Markoff, 1995), although the presence of the N-terminal 26 residues of the NS2a protein of DEN type-2 virus allows correct processing of the precursor (Leblois & Young, 1995). Therefore it is believed that the NS2a protein is required to influence the conformation of the NS1 protein to allow recognition of the cleavage site or greater accessibility by the protease (Leblois & Young, 1995; Falgout & Markoff, 1995).

Two forms of the NS1 protein have been identified in JE, DEN and YF viruses (Mason *et al.*, 1987a; Mason, 1989; Falgout *et al.,* 1989; Chambers *et al.,* 1990b; Konishi *et al.,* 1991). In addition to NS1 a larger protein NS1' has also been identified and is believed to result from an alternative cleavage event in the NS2a protein. The alternative NS2a protein produced by YF virus is thought to derive from hydrolysis at a dibasic cleavage site which requires the action of the NS2b-NS3 protease complex (Nestorowicz *et al.,* 1994). However, the 56 kDa NS1' protein identified for JE virus is predicted to result from an alternative cleavage site upstream of the putative site of YF virus (Mason, 1989; Nestorowicz *et al.,* 1994).

The function of the NS1 protein remains unclear. The E protein is synthesized prior to the NS1 protein but secreted simultaneously suggesting that the NS1 protein is involved in virion assembly or release (Lee *et al.,* 1989). However, the NS1 protein does not associate with mature virions and is found in the same regions of the cell as double stranded RNA, suggesting that the protein is involved in RNA

replication (MacKenzie *et al.,* 1996a). This theory is supported by evidence that mutations in the NS1 protein affect virus replication (Pletnev *et al.*, 1993; Muylaert *et al.,* 1996, 1997).

1.4.2. The NS2b & NS3 Proteins

The NS3 protein is the second largest viral protein with a molecular weight of 67-76 kDa. The protein is highly conserved among flaviviruses and appears to be multifunctional and involved in RNA replication and polyprotein processing.

The C-terminal 149-323 residues of the NS3 protein have been shown to have RNA-stimulated NTPase activity (Wengler & Wengler, 1991; Warrener *et al.,* 1993; Takegami *et al.,* 1994; Kuo *et al.,* 1996) and share sequence similarity with the DEAD family of RNA helicases (Gorbalenya *et al.,* 1989b; Lain *et al.*, 1989; Kadare & Haenni, 1997). This potential helicase function has implicated the NS3 protein in forming part of the RNA replicase complex in association with the NS5 protein. The NS3 and NS5 proteins form a complex which is localized in the proposed site of RNA replication (Edward & Takegami, 1993; Kapoor *et al.,* 1995; Chen *et al.,* 1997). The presence of additional viral proteins is not required for the formation of this complex, although the involvement of cellular proteins cannot be eliminated at this time (Kapoor *et al.,* 1995). The NS3 protein binds specifically to the 3'-NCR of the plus strand viral RNA (Takegami *et al.*, 1994; Chen *et al.,* 1997) and the presence of the NS3 protein is required for RNA synthesis (Bartholomeusz & Wright, 1993; Takegami *et al.*, 1994).

It has been proposed that the C-terminus of the NS3 protein has RNA 5'-triphosphatase activity and is involved in the formation of the type-1 cap at the 5'-terminus of viral RNA (Wengler & Wengler, 1993). The NTPase and triphosphatase active sites are separate, illustrated by the fact that magnesium ions stimulate NTPase activity but inhibit triphosphatase activity (Wengler & Wengler, 1993).

The N-terminal residues of the NS3 protein share sequence similarity with trypsin-like serine proteases (Bazan & Fletterick, 1989; Gorbalenya *et al.,* 1989a). Among

flaviviruses there are four conserved domains in this region: three include the components of the catalytic triad, His-51, Asp-75 and Ser-135 for WN virus; and the fourth contains the putative substrate binding pocket. Deletion analysis and site-directed mutagenesis has shown that the N-terminal 184 residues of the NS3 protein contain protease activity (Chambers *et al.,* 1990c; Preugschat *et al.,* 1990; Falgout *et al.,* 1991; Wengler *et al.,* 1991) and mutations in the putative catalytic triad and substrate binding pocket result in the abolition of this activity (Chambers *et al.,* 1990c; Preugschat & Strauss, 1991; Preugschat *et al.,* 1991; Wengler *et al.,* 1991).

The NS3 protein alone has no protease activity and the presence of the NS2b protein, either *in cis* or *in trans*, is required for mediated cleavage (Preugschat *et al.,* 1990; Chambers *et al.,* 1991; Falgout *et al.,* 1991; Jan *et al.,* 1995). NS2b is a small protein with a molecular weight of 12-15 kDa and although the primary sequence is not highly conserved among flaviviruses there is conservation in the hydrophobicity plots. NS2b is essentially a hydrophobic protein and is presumably membrane associated. Residues 53-92 of the NS2b protein of DEN type-4 virus form a hydrophilic domain, surrounded by hydrophobic regions, which is conserved among flaviviruses (Falgout *et al.,* 1993). This domain is important for protease activity (Chambers *et al.* 1993; Falgout *et al.,* 1993; Jan *et al.,* 1995) and it has been proposed that charged residues within the conserved domain interact with the N-terminal 184 residues of the NS3 protein, thus forming a complex (Arias *et al.,* 1993; Chambers *et al.,* 1993; Jan *et al,*. 1995). The presence of the NS2b protein may result in a conformational change in the NS3 protein which leads to the activation of the protease active site (Falgout *et al.,* 1993).

The NS2b-NS3 protease complex acts in the cytosol and mediates the cleavage events at the NS2a/NS2b, NS2b/NS3, NS3/NS4a and NS4b/NS5 junctions, thus creating the N-termini of the NS2b, NS3, NS4a and NS5 proteins (Chambers *et al.,* 1990c, 1991; Preugschat *et al.,* 1990; Rice & Strauss, 1990; Falgout *et al.,* 1991; Preugschat & Strauss, 1991; Cahour *et al.,* 1992; Zhang *et al.,* 1992). The cleavage site follows two basic amino acids (Arg-Arg, Lys-Arg or Arg-Lys) or in the case of DEN viruses, Gln-Arg, and precedes a short side-chain amino acid (Gly, Ser or Ala) (Mackow *et al.*, 1987; Chambers *et al.,* 1990a).

In addition, the NS2b-NS3 protease has been implicated in the creation of the C-termini of the C protein (Speight & Westaway, 1989; Lobigs, 1993; Yamshchikov & Compans, 1994) and the NS4a protein (Chambers *et al.,* 1991; Cahour *et al.,* 1992; Lobigs, 1992; Lin *et al.,* 1993). The N-terminal cleavage of the NS4b protein is mediated by an ER membrane host protease, presumably signal peptidase (Cahour *et al.,* 1992; Lin *et al.,* 1993). However, NS2b-NS3 mediated cleavage at a site within the NS4a protein is required for cleavage at the NS4a/NS4b junction (Lin *et al.,* 1993).

The NS2b-NS3 protease complex has also been implicated in mediating the internal cleavage of the NS2a and NS3 proteins (Arias *et al.,* 1993; Zhang & Padmanabhan, 1993; Nestorowicz *et al.,* 1994; Teo & Wright, 1997). The internal cleavage site within the NS3 protein lies in the putative RNA helicase motif and therefore may be important in the regulation of virus replication.

1.4.3 The NS4a, NS4b & NS5 Proteins

The function of the NS4a and NS4b proteins is not understood at the present time. The molecular weights of the NS4a and NS4b proteins are 16 kDa and 27 kDa respectively. There is little sequence similarity exhibited between these proteins of different flaviviruses, although the structural features remain similar with several potential membrane spanning domains (Chambers *et al.,* 1990a). The NS4a and NS4b proteins may interact with other viral proteins, e.g. the NS3 or NS5 proteins, thus causing their membrane localization (Rice *et al.,* 1985).

The NS5 protein is the most conserved flavivirus protein and the largest with a molecular weight of 91-104 kDa. The sequence of the NS5 gene extends to the end of the ORF (Castle *et al.*, 1986). There is 45% amino acid sequence similarity between the NS5 proteins of the insect virus Cell Fusing Agent (CFA) and TBE virus, compared with only 20% similarity between the E proteins of these two virus species (Cammisa-Parks *et al.,* 1992).

The C-terminal region of the NS5 protein is particularly conserved and contains four conserved motifs found in RNA-dependent RNA polymerases; including the Gly-Asp-Asp motif at residues 666-668 and the Ser-Gly-$(X)_3$-Thr-$(X)_3$-Asn-Thr motif at residues 603-613 of YF virus, where X denotes any residue (Kamer & Argos, 1984; Rice *et al.,* 1985; Poch *et al.,* 1989).

RNA polymerase activity has been detected in flavivirus infected cell extracts (Chu & Westaway, 1985, 1987; Grun & Brinton, 1986, 1988; Bartholomeusz & Wright, 1993), particularly associated with the ER membrane in the perinuclear region of the cytoplasm (Lubiniecki & Henry, 1974; Grun & Brinton, 1987; Takegami & Hotta, 1989; Edward & Takegami, 1993). The NS5 protein has been detected in the perinuclear region and the nucleus of infected cells (Buckley *et al.,* 1992; Kapoor *et al.,* 1995). Two phosphorylated forms of the NS5 protein have been detected in DEN type-2 virus infected cells, although only one form binds with the NS3 protein to form a complex which is detected predominantly in the perinuclear region of the cytoplasm (Kapoor *et al.,* 1995).

The NS5 protein binds to viral RNA, specifically the 3'-terminal 83 nucleotides of plus strand viral RNA, which forms a stem-loop structure (Tan *et al.,* 1996; Chen *et al.,* 1997). The presence of anti-sera to the NS5 protein inhibits RNA synthesis (Bartholomeusz & Wright, 1993; Edward & Takegami, 1993; Tan *et al.,* 1996). These observations have provided evidence to support the proposed RNA polymerase activity of the NS5 protein. Recently it was reported that the NS5 protein of DEN type-1 virus expressed in *E. coli* exhibits RNA-dependent RNA polymerase activity without cellular or viral co-factors (Tan *et al.,* 1996).

Analysis of sequence data has led to the proposal that the NS5 protein is bifunctional and the N-terminal region encodes motifs conserved in methyltransferases (Koonin, 1993). The conserved domain $(U)_2$-Asp/Glu-X-Gly-X-Gly-X-Gly at residues 77-85 of YF virus, where U denotes bulky aliphatic residues and X denotes any residue, is the putative binding motif of s-adenosylmethionine utilizing methyltransferases (Koonin, 1993). Methyltransferase activity is required for the formation of the cap structure at the 5'-terminus of the flavivirus genome

(Rozanov *et al.*, 1992) and may act in parallel with the putative 5'-triphosphatase encoded within the NS3 protein (Wengler & Wengler, 1993).

1.5. The Noncoding Regions (NCR)

Flanking the ORF are two regions of noncoding nucleotides. In WN virus the 5' and 3'-NCRs are 96 and 571 nucleotides in length respectively (Wengler & Castle, 1986; Castle & Wengler, 1987). Both NCRs have the potential to form secondary structures (Rice *et al.*, 1985; Brinton *et al.*, 1986; Wengler & Castle, 1986; Hahn *et al.*, 1987b; Brinton & Dispoto, 1988; Mandl *et al.*, 1993; Gritsun *et al.*, 1997; Proutski *et al.*, 1997a, b). Flaviviruses lack a polyadenylated tail (Wengler *et al.*, 1978; Wengler & Wengler, 1981) but instead have the conserved dinucleotides 5'-CU-3' at the 3'-terminus (Wengler & Wengler, 1981). These nucleotides are complementary to the 5'-AG-3' motif at the 5'-terminus, where the initial adenine residue forms part of a type-1 cap structure (Wengler *et al.*, 1978; Cleaves & Dubin, 1979; Castle & Wengler, 1987).

Both flanking NCRs are believed to contain component elements important for viral replication and translation of the genome. Point mutations in the 3'-NCR have been associated with altered virulence of YF and JE viruses (Hahn *et al.*, 1987a; Nitayaphan *et al.*, 1990). The 5'-NCR of hepatitis C virus, a member of the family *Flaviviridae*, contains an internal ribosome entry site important for translation of the genomic RNA (Tsukiyama-Kohara *et al.*, 1992). Deletion analysis within the 3' and 5'-NCRs of DEN type-4 virus has illustrated the importance of the NCRs, by producing non-viable virus or virus with lower translation or replication efficiencies (Cahour *et al.*, 1995; Men *et al.*, 1996).

The length of the 3'-NCR varies between and within flavivirus species (Mandl *et al.*, 1991b; Wallner *et al.*, 1995; Poidinger *et al.*, 1996; Wang *et al.*, 1996). This size variability is the result of deletions and insertions in a variable region immediately after the ORF stop codon and is followed by a highly conserved core element which extends over the 3'-terminal 120 and 335 nucleotides in TBE and YF viruses

respectively (Wallner *et al.*, 1995; Wang *et al.*, 1996). The variable region of several strains of TBE virus contain a polyadenylated tract (Mandl *et al.*, 1991b; Wallner *et al.*, 1995). A similar arrangement of variable and conserved regions is found in the 3'-NCR of pestiviruses (Deng & Brock, 1993). It is possible that the variable region acts as a spacer to separate the folded portion of the NCR from the rest of the genome, thus allowing more efficient access and binding with viral and cellular factors (Gritsun *et al.*, 1997).

Computer generated folding patterns and RNase cleavage analyses have demonstrated that the 3'-terminal 90-100 nucleotides form a stem-loop structure which is conserved among the flaviviruses (Grange *et al.*, 1985; Brinton *et al.*, 1986; Takegami *et al.*, 1986; Wengler & Castle, 1986; Mohan & Padmanabhan, 1991) and may form a three dimensional pseudoknot structure in WN virus (Shi *et al.*, 1996a). Recently computer folding analysis has suggested that this terminal secondary structure is one of three independently folded structures within the 3'-NCR (Proutski *et al.*, 1997b). The structures of region I and the terminal region III are conserved among flaviviruses while region II is conserved within flavivirus serocomplex groups. However, the secondary structure predicted for strains of YF virus differ considerably in regions I and III corresponding to the virulence characteristics of the virus strain and result from single point mutations (Proutski *et al.*, 1997a).

The primary sequence of the 3'-NCR is not highly conserved between flaviviruses except for several motifs which are predicted to reside on exposed loops within the secondary structure. The motif 5'-CACAG-3' is conserved among all flaviviruses and present on the 3'-termini of both the plus and minus strands of RNA (Brinton *et al.*, 1986; Takegami *et al.*, 1986; Brinton & Dispoto, 1988). Therefore the motif is a potential signal sequence for a viral or cellular factor involved in replication.

Mosquito-borne flaviviruses contain two conserved regions of approximately 18-20 nucleotides, termed the CS1 and CS2 boxes, which occur within region II of the 3'-NCR (Hahn *et al.*, 1987b; Proutski *et al.*, 1997b). The CS1 box is complementary to a region of the C gene and may function as a cyclization sequence causing the formation of a panhandle structure during replication (Hahn *et al.*, 1987b). The CS2 box is predicted to be exposed on the secondary structure of region II irrespective of

the structural variability of this region, suggesting that this motif is of importance (Proutski *et al.,* 1997b). However, deletion of the CS2 box results in viable DEN type-4 virus (Men *et al.,* 1996). The 3'-NCR of TBE virus contains stretches of homopyrimidine and homopurine nucleotides at the same location as the CS1 and CS2 boxes of mosquito-borne flaviviruses. Sequence stretches of this type have been associated with regulation of translation, RNA stability and replication in other virus species (Wallner *et al.,* 1995).

The NS3 and NS5 proteins, which are believed to form the RNA replicase complex, have been demonstrated to bind specifically with the 3'-NCR illustrating the importance of this region in the initiation of replication (Takegami *et al.,* 1994; Tan *et al.,* 1996; Chen *et al.,* 1997). In addition, a number of cellular proteins have been identified which bind specifically to the 3'-NCR of plus or minus strand RNA (Blackwell & Brinton, 1995; Shi *et al.,* 1996b). Proteins which bind to the 3'-NCR of minus strand RNA fail to bind to the complementary 5'-NCR of plus strand RNA. This is not surprising as the 5' and 3'-NCRs, of both the plus and minus RNA strands, are involved in translation and replication respectively and different cellular factors would be expected to be involved in these processes.

1.6. Flavivirus Replication and Maturation in Host Cells

The method by which virus particles infect cells is not clearly understood and two pathways have been identified. Hase *et al.* (1989a, b) demonstrated that JE and DEN viruses can enter mosquito and mammalian cells by direct fusion with the plasma membrane. However, WN virus is only observed to enter cells by this mechanism if the extracellular medium is mildly acidic and results in non-infectious virions (Kimura *et al.,* 1986; Kimura & Ohyama, 1988). Under conditions of physiological pH WN, KUN and YF viruses enter cells via receptor-mediated endocytosis (Gollins & Porterfield, 1985; Kimura *et al.,* 1986; Ishak *et al.,* 1988; Ng & Lau, 1988). Single virions attach to the plasma membrane which thickens and invaginates to form a vesicle coated in clathrin (Ng & Lau, 1988). The vesicles uncoat and fuse to one

another thus forming prelysosomal endosomes which are found near the perinuclear region of the cell (Ishak *et al.,* 1988; Gollins & Porterfield, 1985).

The mildly acidic conditions within the prelysosomal endosome induce conformational changes in the E protein (Kimura & Ohyama, 1988) which irreversibly alters the oligomeric organization of the E protein on the surface of the virion, from dimers to trimers (Allison *et al.,* 1995). This activates the virion causing fusion with the endosome membrane and subsequent release of the nucleocapsid (Gollins & Porterfield, 1985, 1986a, b; Ng & Lau, 1988).

The cellular receptors involved in flavivirus infection have not been identified and may differ on different cells (Marianneau *et al.,* 1996). JE virus binds to a 74 kDa protein found in the plasma membrane of Vero cells which may form part of the cellular receptor (Kimura *et al.,* 1994).

The released RNA has two functions; replication and translation, which appear to occur in different areas of the cytoplasm (Boulton & Westaway, 1976). The complementary minus strand of RNA is synthesized in the perinuclear region of the cytoplasm to produce a 20S double-stranded RNA replicative (RF) form. Semiconservative replication of the RF produces the heterogeneous replicative intermediate (RI) form from which the newly synthesized 44S single-strand RNA is released (Cleaves *et al.*, 1981; Chu & Westaway, 1985; Westaway, 1987). Only one RNA strand is replicated per cycle which takes approximately 15 minutes (Chu & Westaway, 1985). Replication is controlled by an unknown mechanism which favours the production of plus strand RNA so that minus strand synthesis makes up only 10% of total RNA synthesis (Cleaves *et al.,* 1981). The plus strand RNA is utilized for further translation and encapsidation into new virions.

A characteristic effect of flavivirus infection is the proliferation of the ER and vacuoles (Ng et al., 1983; Ishak *et al.,* 1988; Ng & Hong, 1989). Translation of the genomic RNA, which is the only viral messenger RNA (mRNA) present in infected cells (Stollar *et al.,* 1967; Boulton & Westaway, 1977; Naeve & Trent, 1978; Monckton & Westaway, 1982), occurs in association with the ER and the resulting polyprotein is processed co- and post-translationally as summarized in Fig. 1.1 and discussed below.

The N-termini of the prM, E, NS1 and NS4b proteins are generated by signal peptidase mediated cleavage within the lumen of the ER. Hydrophobic stretches at the C-termini of the upstream proteins, i.e. proteins C, prM, E and NS4a respectively, are required as signal sequences for translocation (Falgout *et al.,* 1989; Markoff, 1989; Nowak *et al.,* 1989; Ruiz-Linares *et al.,* 1989; Depres *et al.,* 1990; Jacobs *et al.,* 1992; Qu *et al.,* 1993; Falgout & Markoff, 1995).

The NS1/NS2a junction is cleaved within the lumen of the ER by an unknown cellular protease (Falgout & Markoff, 1995). The host protease recognizes a unique signal sequence, involving the absence of an upstream hydrophobic signal and the presence of a Met/Leu-Val-X-Ser-X-Val-X-Ala motif at the C-terminal 8 amino acids of the NS1 protein, where X denotes any residue (Hori & Lai, 1990; Pethel *et al.,* 1992).

The NS2b-NS3 protease complex mediates the cleavage events which result in the generation of the N-termini of the NS2b, NS3, NS4a and NS5 proteins (Chambers *et al.,* 1990c, 1991; Preugschat *et al.,* 1990; Rice & Strauss, 1990; Falgout *et al.,* 1991; Preugschat & Strauss, 1991; Cahour *et al.,* 1992; Zhang *et al.,* 1992). In addition, the protease complex mediates the cleavage of anchored-C to virion-C which is essential for processing the structural proteins (Speight & Westaway, 1989; Lobigs, 1993; Yamshchikov & Compans, 1994).

Although the NS4a/NS4b junction is cleaved by signal peptidase in the lumen of the ER (Cahour *et al.,* 1992; Lin *et al.,* 1993), the presence of the NS2b and NS3 proteins is essential for this cleavage event (Preugschat & Strauss, 1991). Upstream of the NS4a/NS4b cleavage site is a conserved viral protease cleavage site, Gln-Arg↓Ser in YF virus, and site-directed mutagenesis in this region also abolishes the NS4a/NS4b cleavage event (Lin *et al.,* 1993). Therefore the viral protease mediated cleavage which generates the C-terminus of NS4a is a prerequisite for the generation of the NS4b N-terminus.

The cleavage of prM to M occurs late in the maturation process immediately before or during virus release (Murray *et al.,* 1993). This cleavage event requires acidic conditions and is believed to occur in the acidic post-Golgi vesicles (Rice *et al.,* 1985; Randolph *et al.,* 1990; Heinz *et al.,* 1994b). The cleavage site is conserved

among flaviviruses and the signal sequence preceding the site is Arg-X-Arg/Lys-Arg where X denotes any residue, which implicates the action of an unidentified host protease (Rice *et al.*, 1985). The pr fragment is released into the extracellular fluid and is not rapidly degraded (Randolph *et al.*, 1990; Murray *et al.*, 1993; Yamshchikov & Compans, 1995).

Assembly of the virion occurs in association with the intracellular membranes and particles are associated with the ER and Golgi apparatus (Filshie & Rehacek, 1968; Boulton & Westaway, 1976; Ng, 1987). The basic residues of the C protein interact with the genomic RNA to form the nucleocapsid. The hydrophobic stretch of anchored-C may localize the nucleocapsid near the ER. Nucleocapsids have only rarely been observed in the cytoplasm suggesting that maturation is a rapid event (Hase *et al.*, 1987a; Chambers *et al.*, 1990a). The prM and E proteins are translocated in the lumen of the ER and formation of the envelope results from budding of the nucleocapsids through the ER membrane (Leary & Blair, 1980). However, nucleocapsids have been observed budding through the plasma membrane thus obtaining an envelope and release from the cell at the same time (Hase *et al.*, 1987a; Ng, 1987; Ng *et al.*, 1994). Virions within the ER pass along the secretory pathway, through the Golgi apparatus and into post-Golgi vesicles which travel to the plasma membrane and release virions by exocytosis (Filshie & Rehacek, 1968; Hase *et al.*, 1987b; Ishak *et al.*, 1988). Another possible mechanism of virus release involves lamellae of smooth membranes opening directly to the exterior of cells (Westaway, 1980).

1.7. Louping-ill Virus

A disease of sheep known as louping-ill has been recognized in the Borders of Scotland for at least 200 years. However, several conditions were described by the term louping-ill, leading to difficulties in investigating and treating the problem (Laing, 1832; Tod, 1832). McFadyean (1894, 1900) identified three conditions frequently described as louping-ill; pyaemic meningitis, gastritis or enteritis, and

encephalitis or encephalomyelitis. The latter describes true louping-ill which is now known to occur in many upland areas of Scotland, Northern England, Wales, South-West England and Ireland (Hughes & Kershaw, 1957; Reid, 1988). The name louping-ill derives from one of the symptoms of the disease whereby infected animals leap into the air when handled or alarmed.

It was not until 1930 that successful, unambiguous transmission of the disease between sheep was achieved by inoculation of infected material (Pool *et al.,* 1930; Greig *et al.,* 1931) and the aetiological agent identified as a filterable virus (Gordon *et al.,* 1932). The involvement of the sheep-tick, *Ixodes ricinus*, had long been implicated as the vector of the virus and this was proved in 1932 (MacLeod & Gordon, 1932).

LI virus was estimated to have a diameter of 15-20 nm by ultrafiltration (Elford & Galloway, 1933; Tang *et al.,* 1937) and was shown to be sensitive to formalin, bile salts and ethyl ether (Gordon *et al.*, 1932; Smith, 1939; Andrewes & Horstmann, 1949). Antigenic analysis identified LI virus as being closely related but distinct from the Western and Far Eastern subtypes of TBE virus (WTBE and FETBE respectively) (Casals & Webster, 1944; Edward, 1950).

LI virus was the first tick-borne virus to be isolated and a vaccine was quickly developed (Gordon, 1934). Nevertheless, the virus and its associated disease have persisted in the British Isles.

1.7.1 *Ixodes ricinus*, the Vector of Louping-ill Virus

Although 15 species of *Ixodes* exist in the British Isles only *Ixodes ricinus* has been implicated in the transmission of LI virus. However, *Rhipicephalus appendiculatus*, a species of tick from Africa, can experimentally transmit LI virus (Alexander & Neitz, 1933). *I. ricinus* is a hard tick with a three host life-cycle and lives for 2-4 years (Milne, 1944). Ticks only spend approximately three weeks of their life on hosts and the rest of the time is spent among vegetation (MacLeod, 1932). Therefore there are two factors affecting the tick population and thereby affecting the virus

population; the availability of hosts and the suitability of the habitat. *I. ricinus* ticks are very susceptible to desiccation and unfed and engorged ticks require a relative humidity above 92% and 95% respectively (Lees, 1946). Suitable conditions are found within the layer of decaying vegetation near the soil and the relative humidity is proportional to the thickness of this mat which in turn correlates with the tick population (Milne, 1944, 1952).

Originally, much of upland Britain was covered in forest where *I. ricinus* would have existed among the leaf litter. Most of this habitat was destroyed to make room for agriculture, and resulted in the creation of grassland and moorland. The dense matted layer of decaying vegetation, which is found in abundance on moorland, provides an ideal habitat for *I. ricinus*. In addition, the introduction of large populations of susceptible sheep hosts enabled the tick population to expand together with any tick-borne pathogens carried by these arthropods, e.g. LI virus and *Erhlichia phagocytophila*, the causative agent of tick-borne fever.

I. ricinus exhibits seasonal activity and becomes active when air temperatures exceed 7°C (MacLeod, 1932; Milne, 1945a). In Ayrshire, the nymphal stage is active during April and May and the larval stage is also active in May (Smith *et al.,* 1964a). Adults do not appear to have such a marked peak of activity. In western areas of Britain and Ireland tick activity is bimodal with an additional peak of activity in the autumn. Whether or not this peak represents a separate population of ticks has been debated (Campbell, 1952; Gray, 1982).

Each instar of *I. ricinus* is active for about 30 days during which time ticks attempt to locate a host by questing (Milne, 1945b). Throughout this active period ticks travel vertically to the tips of vegetation and await a passing host. To prevent desiccation ticks must frequently return to the vegetation mat to restore their humidity balance and therefore during the active period ticks will only spend on average nine days at the tip of vegetation (Lees & Milne, 1951).

Temperature, warm air currents and carbon dioxide stimulate *I. ricinus* to attach themselves to a passing animal (Lees & Milne, 1951). Therefore ticks are very cosmopolitan in the species they infest and larvae and nymphs have been observed on mammals, birds and reptiles (Milne, 1949). Adult *I. ricinus* are usually associated

with larger mammals where the males do not feed but attempt to find a mate. In upland grazing areas the tick population can be almost exclusively supported by sheep (Milne, 1949). Over 95% of adult females are believed to feed on sheep in some areas, as well as 80-90% of nymphs. However, in many areas other species have been shown to harbour large populations of ticks e.g. red deer (*Cervus elaphus*) and mountain hares (*Lepus timidus*).

Transovarial transmission of LI virus has not been observed and therefore persistence of the virus most likely depends upon transstadial transmission (MacLeod & Gordon, 1932). Ticks infected as larvae or nymphs transmit the virus during the next instar as nymphs and adults respectively, while the uptake of virus by adult female ticks is of no epizootiological importance. When ticks moult many physiological changes occur producing conditions not ideal for virus survival. Therefore, although 100% of larvae may take up LI virus when feeding on an infected animal, only 10% of the resulting nymphs may harbour detectable virus (Beasley *et al.,* 1978). As a result the incidence of LI virus infection in ticks in enzootic areas appears to be low, at less than one in 600 (Varma & Smith, 1971).

Experimental infections of a wide range of animals with LI virus result in variable viraemia titres. It has been experimentally shown that for *I. ricinus* nymphs and larvae to take up the virus in a blood meal the titres of virus must exceed 10^3 and 10^4 plaque forming units (p.f.u.) per 0.2 ml respectively (Beasley *et al.,* 1978). In the majority of species experimentally infected with the virus the levels of viraemia did not approach these threshold-figures (Reid, 1988). However, recent research provides evidence that LI virus can be transmitted between ticks co-feeding on mountain hares. In co-feeding experiments on uninfected animals naive nymphs become infected by feeding near an infected adult *I. ricinus*, even though viraemia levels never exceeded the threshold level discussed above. Low level transmission by co-feeding was also detected when ticks fed on seropositive mountain hares with no circulating virus (Jones *et al.,* 1997). These results are important in the epizootiology of the virus as they indicate that sheep may not be the only important species in the maintenance of the virus. A similar set of experiments carried out on red deer provided no evidence of transmission by co-feeding, suggesting that red deer

are of importance only in the amplification of *I. ricinus* numbers (Jones *et al.,* 1997). This conclusion is supported by the failure of LI virus to persist on the Isle of Rhum after sheep were removed even though there was a large population of resident red deer to maintain the tick population (Adam *et al.*, 1977).

1.7.2. Vertebrate Hosts

Although LI disease is usually associated with sheep the virus also causes disease in other species of domestic animals, for example; cattle (Fletcher, 1936; Walton & Kennedy, 1966), pigs (Bannatyne *et al.,* 1979; Ross *et al.,* 1994), goats (Gray *et al.,* 1988), horses (Fletcher & Galloway, 1937; Timoney *et al.,* 1976), dogs (MacKenzie *et al.,* 1973) and captive bred roe and red deer (Reid *et al.,* 1976, 1978a). The incidence of disease in these animals is sporadic, probably due to the practice of few domestic animals other than sheep being grazed on upland pasture.

In Ireland the relative air humidity levels are higher than in Britain and this results in a wider distribution of *I. ricinus* and LI virus. This distribution includes good quality lowland pastures, where more domestic species come into contact with both *I. ricinus* and LI virus (Walton & Kennedy, 1966; Walton, 1967). Therefore in Ireland more species are involved in the transmission cycle of the virus, while in Britain an almost exclusive tick-sheep cycle can exist.

Two serological surveys of LI virus infection in red deer have been undertaken. In the earlier study 37% stags and 22% hinds from across Scotland were seropositive, while in the later study a total of 29.4% red deer had seroconverted (Dunn, 1960; Adam *et al.,* 1977). However, there has been no recorded incidence of naturally occurring disease in wild red deer. Studies of small mammals have shown that wood mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*) and common shrews (*Sorex araneus)* can be infected with the virus, although it is not possible to deduce from these experiments if a debilitating disease resulted from these infections (Smith *et al.,* 1964b; Kaplan *et al.,* 1980).

Natural infection of avian species with LI virus has only been reported in wild and captive bred red grouse, *Lagopus lagopus scoticus* (Watt *et al.,* 1963; Williams *et al.,* 1963; Timoney, 1972; Reid & Boyce, 1974). Eighty percent of experimentally infected grouse died as a result of the virus (Reid, 1975). In subsequent studies comparing the breeding success of wild grouse in heavily tick infested areas and relatively tick free areas, a higher chick mortality was observed in the former with only three out of 42 chicks surviving (93% mortality) to adulthood (Duncan *et al.,* 1978; Reid *et al.,* 1978b). For a grouse population to survive each hen must rear at least 2-3 young per year. In this study area the rearing rate was only 0.6 young per hen, which would result in a population crash.

It has recently been shown that chicks can become infected with LI virus via the oral route with the ingestion of infected *I. ricinus* (Jones, pers. com.). Although adult grouse eat heather young chicks feed on arthropods. The feeding requirements of the chicks brings broods into marshy areas where ticks and insects are abundant, thus also increasing the risk of infection by the tick-bite route.

Humans are susceptible to LI virus infection, although the resulting disease is generally considered not to be too severe (Davidson *et al.,* 1991). However, several severe cases of the disease have been reported, including one fatality (Williams & Thorburn, 1962). Between 1934-1991 there was a total of 45 reported cases of disease in man of which 26 resulted from laboratory infections (Rivers & Schwentker, 1934: Davison *et al.,* 1948; Edward, 1948a; Brewis *et al.,* 1949; Lawson *et al.,* 1949; Likar & Dane, 1958; Ross, 1961; Williams & Thorburn, 1962; Cooper *et al.,* 1964; Schonell *et al.,* 1966; Webb *et al.,* 1968; Reid *et al.,* 1972; Davidson *et al.,* 1991). Few of the naturally occurring cases have been associated with tick bites. Farmers, shepherds and other people involved in the slaughter of animals are apparently more at risk of developing LI virus infections than people working with live animals. This is probably due to virus aerosols being dispersed when infected animals are killed or dissected. Other cases of the disease cannot be attributed to tick-bites or occupational risks and the source of the virus cannot be identified. However, piglets fed with infected lambs' meat developed louping-ill disease (Bannatyne *et al,* 1979) and LI virus has been recovered from the milk of ewes and

goats (Gresikova *et al.,* 1961; Reid *et al.,* 1984; Reid & Pow, 1985), suggesting the possibility of transmission by the oral route.

1.7.3. Louping-ill Encephalomyelitis

The typical disease in sheep is biphasic. The virus initially replicates outside the central nervous system (CNS), probably in the regional lymph nodes (Malkova, 1968). Viraemia is first detected 24 hours after infection. Maximum titres in the blood of 10^3-10^7 p.f.u. per 0.2 ml were detected on days 3 and 4 post inoculation (p.i.). This stage of the illness is associated with pyrexia, anorexia and dullness (Reid & Doherty, 1971a, b).

The virus crosses the blood-brain barrier and infects the spinal cord and brain. Under these circumstances the disease may enter the second, encephalitic phase resulting in neurological symptoms which include; ataxia, stiffness of the neck, trembling of limbs, nervous excitability and the characteristic leaping behaviour observed when the animals are handled or alarmed. Paralysis develops, especially in the hind limbs, followed by coma and death. Histopathological analysis of the CNS of infected animals demonstrates that most damage occurs in the spinal cord and brain stem with less pathology observed in other areas of the brain including the Purkinje cells of the cerebellum (Brownlee & Wilson, 1932; Doherty & Reid, 1971a, b). Typical lesions involve necrosis of neurones due to the cytopathic effect of virus replication and also the damaging effect of the inflammatory response (Doherty & Reid, 1971a, b).

A high proportion of sheep and other animals which do not exhibit neurological symptoms after experimental infection with LI virus have detectable lesions in the CNS (Doherty & Reid, 1971a; Reid *et al.*, 1981, 1982). However, these lesions are not as severe as those observed in animals which succumb to the disease. Whether or not a LI virus infection of sheep develops into the often lethal, encephalitic phase depends upon the appearance and increasing titres of neutralizing and HI antibodies (Smith *et al.,* 1964a; Reid & Doherty, 1971a, b). Rising antibody titres are

associated with a decline in viraemia which in turn limits the neurological damage sustained by the animal. By day 4 post experimental infection, titres of virus fell in 73% of sheep that did not progress to the encephalitic phase of the disease, but in only 43% of sheep that went on to develop neurological symptoms (Reid & Doherty, 1971b).

The incidence of encephalitic symptoms in sheep may be increased by several factors, including; stress, nutritional status, age, cold, exercise and concurrent infection with *Erhlichia phagocytophila*, a Rickettsial parasite that is the causative agent of tick-borne fever (Gordon *et al.,* 1932, 1962; MacLeod & Gordon, 1932; Brodie *et al.,* 1986). The effect of *E. phagocytophila* results from the parasites immunosuppressive action (Brodie *et al.,* 1986). While the incidence of LI virus in *I. ricinus* is at a low level, below one in 600 (Varma & Smith, 1971), 44% of nymphal and 32% of adult *I. ricinus* from a study site in Ayrshire were found to be infected with *E. phagocytophila* (Webster & Mitchell, 1989). The presence of *E. phagocytophila*, which alone is usually benign, is also associated with infection of sheep with *Straphylococcus aureus*, the causative agent of tick pyaemia (Brodie *et al.*, 1986). This bacterium usually exists as a commensal organism of the skin, but in the presence of *E. phagocytophila* it can infect joints, internal organs and the spinal cord. A wide range of symptoms are exhibited by sheep suffering from tick pyaemia, including several which are also observed in sheep infected with LI virus (Foggie, 1962). Therefore the presence of tick-borne fever can complicate epizootiological studies of LI virus, especially prior to its discovery in 1932 (McFadyean, 1900; Gordon *et al.,* 1932). *E. phagocytophila* infection also results in the increased incidence of pneumonic pasteurellosis and mycotic infection by the branching fungal hyphae *Rhizomucor pucillus* (Brodie *et al.*, 1986).

Symptoms observed in man are similar to those described above for sheep. The first phase of the disease is associated with influenza-like symptoms (Davidson *et al.*, 1991). Biphasic encephalitis has been most frequently observed but four of five cases reported in Ireland and one case in Scotland resulted in poliomyelitis-like symptoms (Likar & Dane, 1958; Davidson *et al.,* 1991).

Histological studies of the effect of LI virus on the brains of red grouse revealed very different effects from those observed in sheep. The virus causes most pathology in the forebrain of grouse, as opposed to the hind brain of sheep (Buxton & Reid, 1975).

1.7.4. Passive Immunity and Vaccination

It is common practice on LI virus infected farms for the sheep flocks to be sold with the land. Introduction of new sheep from uninfected areas can result in high mortality rates (Gordon *et al.,* 1932; Walton & Kennedy, 1966; Brotherston *et al.,* 1971). However, sheep that survive infection by LI virus are immune for life, and this immunity is passed onto lambs as colostrum antibody. The passive immunity lasts for approximately six weeks and will protect lambs during the first season of tick activity, after which time the lambs are susceptible to the virus (Wilson & Gordon, 1948). Therefore by keeping the same flock of sheep on a farm the mortality rates will be limited, although without vaccination and tick control measures, some hoggs (one year old sheep) will be susceptible to the virus during the next season of tick activity.

Vaccination of sheep with live LI virus was investigated but resulted in high mortality rates (Gordon *et al.,* 1932). An inactivated virus vaccine was quickly developed which consisted of formalin-treated brain, spinal cord and spleen from experimentally infected sheep (Gordon, 1934; Gordon *et al.,* 1962). The vaccine did not induce detectable levels of antibody but sensitized the immune system of sheep against LI viral infections (Williams & Thorburn, 1961; Smith *et al.,* 1964a). Thereby, when naturally infected with LI virus, antibodies appear more rapidly in the serum of vaccinated sheep than in nonvaccinated animals and more speedily reduce the viraemia titres. This limits the potential risk of the virus entering the CNS and causing the lethal, encephalitic phase of the disease. However, levels of viraemia in the blood of sheep still exceeded the threshold levels for transmission to ticks (Beasley *et al.,* 1978) and therefore the vaccination regime had little effect on the

epizootiology of the virus (Smith *et al.,* 1964a; Walton, 1967). The second batch of vaccine distributed commercially was found to be infected with the causative agent of scrapie. The presence of other pathogens is a major disadvantage with the use of infected sheep brain material.

Several alternative vaccines have been developed and tested but are not currently in use. These included formalin-treated infected chick embryo tissue (Edward, 1947, 1948b) and live preparations of attenuated Langat (LGT) and WTBE viruses which are antigenically closely related to LI virus (O'Reilly *et al.,* 1965; Mayer *et al.,* 1969).

In 1967 the formalin-treated sheep brain vaccine was taken off the market because of the high incidence of infection in workers preparing the vaccine. It was not until 1969 that another vaccine was developed, consisting of virus propagated in sheep kidney cells. The virus was then inactivated in formalin before purification in methanol and incorporation into an oil-based adjuvant (Brotherston & Boyce, 1969, 1970; Brotherston *et al.,* 1971). A two dose strategy is optimal and induces high levels of HI and neutralizing antibodies and protects sheep against the initial amplification of the virus (Shaw & Reid, 1981). Titres of circulating antibodies in ewes are sufficiently high to provide passive protection to the next generation of sheep through colostral antibodies. The method of vaccine preparation has been altered for commercial production and the virus is propagated in BHK-21 cells before purification by ultracentrifugation (Shaw, 1981; Reid & Pow, 1995).

The LI virus vaccine is not licensed for human use. However, the vaccine for the closely related TBE virus provides protection against LI virus infection (Gould, pers. com.)

1.7.5. Antigenic Variation Among Strains of Louping-ill Virus

Several studies have reported antigenic variation among LI viruses with varying results. In two studies haemagglutinin activity could only be demonstrated in a portion of the viruses sampled, suggesting strain variation did exist (Williams *et al.,* 1963; Timoney 1971). However, the variation observed may have been due to

limitations in the techniques used in the studies. Clarke (1964) performed HI tests on three strains of LI virus from Scotland and Wales and detected no antigenic variation between the viruses. In a later study of 21 LI viruses all appeared to be identical to the prototype virus, LI/31, with respect to plaque morphology, neutralization and HI activity and mouse pathogenicity (Reid, 1984). However, two LI viruses, SB/526 and SB/527 exhibited different pathogenicity in sheep after subcutaneous inoculation (Reid & Doherty, 1971a). In addition, Gresikova and co-authors (1961) reported the occurrence of an attenuated strain of LI virus.

Hussain (1990) investigated variation among 18 LI viruses from Norway, Britain and Ireland using MAbs. Variation was detected with the identification of antibody escape variants, viruses which are not recognized by specific neutralizing or HI antibodies. This variation was shown to be directly correlated with plaque morphology and virulence in mice. Subsequent work analyzed sequence data for the E genes of 11 LI viruses (Gao, 1995). Geographic variation was observed and it is now possible to use these data to investigate the evolution of LI virus in the British Isles.

1.8. Evolution of the Flaviviruses

The advent of molecular biology, especially sequence analysis, has extended the methodology available to investigate the evolution of viruses. RNA viruses evolve at a faster rate than DNA viruses, with an average of 10^{-4} substitutions per incorporated nucleotide as opposed to 10^{-10} substitutions per incorporated nucleotide for the DNA viruses (Holland *et al.*, 1982). This is due to the lack of proof-reading exonucleases in RNA viruses which correct mismatched base pairs in newly synthesized DNA strands (Holland *et al.*, 1982; Reanney, 1982). Lack of proof-reading results in high levels of non-infectious progeny in RNA viruses which is compensated by the high replication rates and high yields. However, a pool of virus will contain a population of viruses with similar but not identical sequences, a quasispecies population.

Arthropod-borne RNA viruses evolve at a slower rate than those without vectors, e.g. polio, influenza and human immunodeficiency (HIV) viruses, because of the constraining effect imposed by the two host system, the invertebrate vector and the vertebrate host (Nottay *et al.*, 1981; Buonagurio *et al.*, 1986; Hahn *et al.*, 1986). The arthropod and vertebrate hosts each impose their own selective pressures, which may involve reversion when the virus returns to the arthropod from the vertebrate and *vice versa*. Evolution of the virus genome is therefore constrained by the requirements of the two hosts (Clewley *et al.*, 1977; Weaver *et al.*, 1991).

All positive strand RNA viruses have an RNA-dependent RNA polymerase encoded within their genome. Phylogenetic analysis of these proteins have been used to investigate the evolution of RNA viruses at a very broad level (Bruenn, 1991; Koonin, 1991; Blok *et al.*, 1992). These phylogenetic analyses implied that flaviviruses are closely related to the other genera within the family *Flaviviridae*, i.e. pestiviruses and hepatitis C virus, as well as plant luteoviruses, e.g. barley yellow dwarf virus, but are only distantly related to alphaviruses. Phylogenetic analysis using the RNA helicase gene, NS3 in flaviviruses, identified similar relationships (Ohba *et al.*, 1996). However, studies reported by different research groups produced different phylogenies. A later study which investigated the validity of the classification of RNA viruses using RNA-dependent RNA polymerase or helicase genes, implied that such analyses were unsuitable for investigating such divergent viruses, due to the lack of sequence similarities and therefore the loss of phylogenetic information (Zanotto *et al.*, 1996a). The analysis was unable to find conclusive evidence to support a common ancestry for genera within the family *Flaviviridae*. More convincing evidence of relationships between virus groups was found by identifying similarities in the general organization of the viral genomes and the mechanisms used to express genes (Zanotto *et al.*, 1996a).

Porterfield (1980) suggested that all flaviviruses may have a common origin and therefore this supports the concept of a monophyletic group of flaviviruses. This was later also supported by the broad antigenic cross-reactivity found among flaviviruses using E protein-specific MAbs (Gould *et al.*, 1985). Antigenic clusters of flaviviruses were identified by cross-neutralization tests using polyclonal antisera

(Madrid & Porterfield, 1974; Calisher *et al.*, 1989) and these groupings were supported to a large extent by phylogenetic analysis (Mandl *et al.*, 1989b; Blok *et al.*, 1991, 1992; Poidinger *et al.*, 1996). Phylogenetic analysis of the flavivirus E gene provides similar information to analysis of the NS5 gene (Blok *et al.*, 1992; Marin *et al.*, 1995b). There is no evidence of recombination among the flaviviruses and individual phylogenetic analysis of all ten genes and the NCRs results in similar tree topology (Blok *et al.*, 1992; Gritsun *et al.*, 1997).

The mosquito virus CFA has the same genomic organization as flaviviruses and on the basis of comparative sequence alignments and phylogenetic analysis is either the most divergent flavivirus or the only recognized member of a closely related separate genus (Cammisa-Parks *et al.*, 1992; Marin *et al.*, 1995b). Therefore the flaviviruses may have evolved from insect viruses.

The oldest divergence event within the genus *Flavivirus* is the separation of mosquito-borne and tick-borne virus lineages (Fig. 1.2) and thereafter the two virus types have evolved differently (Blok & Gibbs, 1995). The estimated rate at which synonymous (silent) nucleotide substitutions occur does not differ significantly between the mosquito-borne and tick-borne flaviviruses. However, the estimated rate at which nonsynonymous (result in amino acid change) nucleotide substitutions occur in mosquito-borne flaviviruses is almost twice as fast as that observed with tick-borne flaviviruses (Marin *et al.*, 1995b; Zanotto *et al.*, 1995). In addition, amino acid substitutions in tick-borne flaviviruses are more conserved with a high frequency of substitutions for residues with similar physicochemical properties (Zanotto *et al.*, 1995). This probably results from strong purifying selection at the amino acid level due to the constraining effect of the tick vector.

Mosquitoes have a short lifespan and can feed on several hosts. They take in a small volume of the infectious bloodmeal and then replicate the virus to very high titres within a few weeks. This high turnover leads to rapid evolution of the transmitted viruses. On the other hand, ticks have a generation time of months or years during which time they only take three blood meals and therefore the replication cycle of the viruses is slower. The tick-borne flavivirus must survive inside the tick for long periods of time between feeds. This period may be over a

year, during which time the host moults to the next instar when many physiological changes occur (Sonenshine, 1991). Therefore replication in tick tissues has a significantly greater constraining effect on virus evolution than that of the mosquito transmitted viruses.

Due to the evolutionary constraints imposed by the tick vector and its restricted movement the tick-borne flaviviruses have gradually evolved across the northern hemisphere which can be seen as an asymmetrical branching pattern on a phylogenetic tree depicting their evolutionary relationships, Fig. 1.2 (Zanotto *et al.,* 1995). This observation supports the hypothesis that a tick-borne flavivirus cline exists across Europe and Asia, with the more ancient species occurring in the East and the most recently diverged flaviviruses occurring in the West, with LI virus at the extreme end of this cline (Zanotto *et al.,* 1995). Several "corridors" of virus evolution can be seen in this cline, with KFD, LGT, Turkish sheep encephalitis (TSE), Spanish sheep encephalitis (SSE) and LI viruses evolving into India, Malaysia, Turkey, Spain and the British Isles respectively. How an individual species of flavivirus has then further evolved is not yet known.

Tick-borne sheep encephalomyelitic viruses have been reported in Spain, Britain, Ireland, Norway, Turkey, Greece and Bulgaria (Pavlov, 1968; Hartley *et al.*, 1969; Papadopoulos *et al.*, 1971; Gonzalez *et al.,* 1987; Reid, 1988; Gao *et al.,* 1993a, b; Gao, 1995; Marin *et al.,* 1995a,). Antigenic analysis implies that these viruses are very closely related and virtually indistinguishable. Sequence data are now available for viruses from several countries and multiple alignments of these sequences have shown that the strains of LI virus in the British Isles and Norway can be distinguished from all other LI-like viruses. SSE virus is a distinct virus and Greek goat encephalitis (GGE) virus appears to be a strain of TSE virus (Marin *et al.,* 1995a), which is again distinct from other sheep encephalomyelitic viruses (Gao *et al.,* 1993a; Whitby *et al.,* 1993b).

The TBE serocomplex cline involves several "corridors" created as distinct viruses have evolved into new areas, such as Britain, Spain and Turkey (Zanotto *et al.,* 1995). If several viruses, in different locations, independently developed a similar sheep-tick life-cycle then parallel evolution of phenotypic characteristics would be

expected to occur thus resulting in similar antigenic properties being present in distinct viruses.

Recently an attempt was made to estimate the date of divergence of lineages within the *Flavivirus* genus (Zanotto *et al.,* 1996b). Because the approximate dates of isolation were available for many viruses included in the study, an estimate of the average rate of nucleotide substitutions could be calculated. By assuming the rate was constant the level of sequence difference between viruses could be extrapolated to estimate when the lineages, which the viruses represent, diverged.

Zanotto *et al.* (1996b) estimated the rate of nonsynonymous substitutions to be 7.5 x 10^{-5} substitutions/site/year for mosquito-borne flaviviruses. Only limited data were available for estimating the nonsynonymous substitution rates for tick-borne flaviviruses which resulted in a value of 7.28 x 10^{-5} substitutions/site/year. Because the authors had previously shown that the tick-borne flaviviruses are only evolving at 0.56 times the speed of the mosquito-borne flaviviruses (Zanotto *et al.,* 1995), the tick-borne flavivirus nonsynonymous substitution rate was estimated as 4.12 x 10^{-5} substitutions/site/year. The resulting calculations of lineage divergence times estimated that the recognized tick-borne flaviviruses have evolved during the past 2,000 years. According to the estimates, the TBE complex of flaviviruses only reached Western Europe approximately 500 years ago and LI virus evolved during the past 300 years. Although it is recognized that the estimates have relatively large standard errors the values of lineage divergence times strongly suggest that the evolution, of at least the TBE serocomplex of the flaviviruses, is a relatively recent event.

The British Isles have been isolated from mainland Europe since the formation of the English Channel at the end of the last ice age, approximately 8,000 years ago. Therefore LI virus must have been introduced to the British Isles, from mainland Europe, within the last 8,000 years. The high mortality observed in LI virus infections of red grouse suggests that the two species have not evolved together (Reid, 1975) and this provides indirect evidence that LI virus was only recently introduced into the habitat now characteristically associated with this virus.

The phylogenetic analysis appears to contradict the traditional hypothesis that LI virus or its ancestor originated from forests in Britain and emerged onto moorland, with *I. ricinus,* when this habitat replaced woodland in many upland areas of the British Isles. LI virus populations on moorland remained low until the introduction of intensive sheep farming in these areas which resulted in an exponential increase in the tick population, and thereby of the tick pathogens. The fact that woodland game birds; capercaillie, pheasant and black grouse, are resistant to experimental LI virus infection provides evidence to support the theory of the ancestral LI virus initially occurring in the British forest environment (Reid *et al.,* 1980, 1983; Reid & Moss, 1980). However, systematic sampling of woodland dwelling animals has only detected antibodies to LI or TBE virus in rodents living at the forest fringes near moorland. In addition, no flavivirus other than LI virus has ever been isolated in the British Isles. Furthermore, of the three woodland game birds species investigated only black grouse are native to the British Isles. Pheasants and capercaillie were introduced from Asia and Scandinavia respectively where TBE serocomplex viruses also exist in the forest ecosystem. This may account for the genetic resistance among these birds.

The method by which LI virus reached the British Isles has not yet been identified, nor is it known how LI virus evolved onto the moorland habitat while other TBE viruses exist in the forest environment. It is hoped that continued phylogenetic analysis will help to address these issues.

1.9. Aims of the Investigation

Until recently, it was difficult to distinguish fine differences between antigenically closely related viruses. However, with the advent of RT-PCR and sequencing this is no longer a problem. For a long time LI virus was considered to be a single virus species exhibiting little heterogeneity but recent evidence suggests that this may not be the case because of genetic variation. The studies of LI virus variation have been limited in both the number of viruses analyzed and the methodology used. As a

consequence they have raised many questions but provided few answers. One of the major aims of this thesis will therefore be to establish appropriate sequencing methods to carry out a more detailed analysis of LI virus variation in the British Isles and to attempt to identify their spatial and temporal distribution.

Two approaches will be adopted; in the first, comparative alignments will be constructed from sequence data encompassing the complete E gene of a number of LI viruses. The E gene was chosen because it is a good phylogenetic indicator and of considerable biological importance, being involved in receptor binding, membrane fusion and viral virulence. Since analysis of the complete gene can only involve a limited number of viruses, a larger analysis will be carried out by sequencing a well defined region within the E gene of 43 LI viruses from many of the areas in the British Isles where LI disease has been reported. To extend these analyses sequence data from previously investigated LI viruses will be incorporated into the alignments as will the sequences of closely related TBE serocomplex viruses. These alignments will be used to construct phylogenetic trees to investigate the spatial distribution of LI virus.

These sequence data will also be used in an attempt to investigate the temporal dynamics of LI virus distribution and thereby construct a time-frame for the evolution of LI virus in the British Isles. By combining the predicted temporal and spatial elements of LI virus variation the evolution of LI virus in the British Isles may be investigated. The traditional theory of LI virus evolution predicts that the ancestral virus was originally present in the British forests. If this is correct then one might expect different lineages of this ancestral virus to have emerged separately onto moorland pastures in different areas of the country, resulting in distinct viruses causing encephalomyelitis occurring in the British Isles. However, initial data suggest that this is not the case and Zanotto *et al.* (1996b) estimated that LI virus has evolved during the past 300 years. Many historical records are available for this time period which when combined with the molecular data may elucidate the origin of LI virus and its dispersal pattern.

Unlike other tick-borne flaviviruses, which cause significant disease in humans, LI virus is predominantly found in the natural environment existing within the sheep-

tick cycle. Therefore the analysis of genetic determinants of LI virus virulence can be investigated in the natural system and provide a potential model for investigating the pathogenicity of other flaviviruses. The E protein has been identified as a major determinant of flavivirus virulence *in vivo* and virus cytopathogenicity in cell culture. Therefore an additional aim of the thesis will be to investigate the biological properties of LI viruses to elucidate any correlation between genetic variation in the E gene and virulence characteristics. The biological properties of LI viruses will be investigated *in vitro* and *in vivo* by plaque morphology studies and pathogenicity studies carried out in the mouse model. In view of the fact that previously identified escape variants of LI virus have exhibited altered virulence characteristics dependent on the amino acid substitution altering the phenotype of the virus (Jiang *et al.*, 1993), particular attention will be made to any naturally occurring escape mutants identified in this study. The occurrence of LI virus escape variants will be investigated in this thesis in an attempt to further the understanding of their selection and importance on the epizootiology of the virus species as a whole.

A further aim of this thesis will be to continue the analysis of the genetic markers present within the E gene. Three genetic markers have been identified to date which can be used to distinguish between flaviviruses (Shiu *et al.*, 1991, 1992;. Gao *et al.*, 1993a). However, the stability of these markers within a virus species has not been investigated. The total analysis of over fifty LI viruses may establish their reliability and potential as genetic markers in the study of flavivirus classification.

LI virus was the first tick-borne virus to be isolated but has largely been ignored by virologists due to the low incidence of human disease. However, although a sheep vaccine has been in use for more than 60 years the virus still persists in the wild and with the increasing popularity of outdoor pursuits, such as hill-walking, the incidence of human disease through tick-bites may rise. Therefore a proper understanding of the epizootiology of the virus, including the level of variation across the British Isles, is important. It is the hoped that this thesis will answer a number of questions on the biology of LI virus raised by previous studies and provide a fuller understanding of the variation among LI virus in the British Isles which can be used to investigate the evolution of the virus.

Serocomplex Group	Type Species	Host Species	Geographic Distribution	Additional important flaviviruses
1	Tick-borne Encephalitis (TBE) virus	tick-borne	Europe & Asia	Louping-ill (LI) virus Kyasanur Forest Disease (KFD) virus Omsk haemorrhagic fever (OHF) Powassan (POW) virus
2	Rio Bravo virus	bats	Africa	Apoi virus Saboya virus
3	Japanese Encephalitis (JE) virus	mosquito-borne	cosmopolitan	St. Louis Encephalitis (SLE) virus West Nile (WN) virus Kunjin (KUN) virus
4	Tyuleniy (TYU) virus	tick-borne	coasts	Saumarez Reef (SRE) virus Meaban (MEA) virus
5	Ntaya virus	mosquito-borne	cosmopolitan	Bagaza virus
6	Uganda S virus	mosquito-borne	Africa	Banza virus Edge Hill virus
7	Dengue (DEN) virus	mosquito-borne	cosmopolitan	Dengue viruses type 1-4
8	Modoc virus	rodents	North America	Cowbone Ridge virus
9	Unassigned group	tick-borne, mosquito-borne & no-known vectors	cosmopolitan	Yellow Fever (YF) virus Wesselsbron virus

Table 1.1. Serocomplex categorization of the flaviviruses. The table includes the vector or host species involved and the geographic distribution of the flaviviruses within the serocomplex.

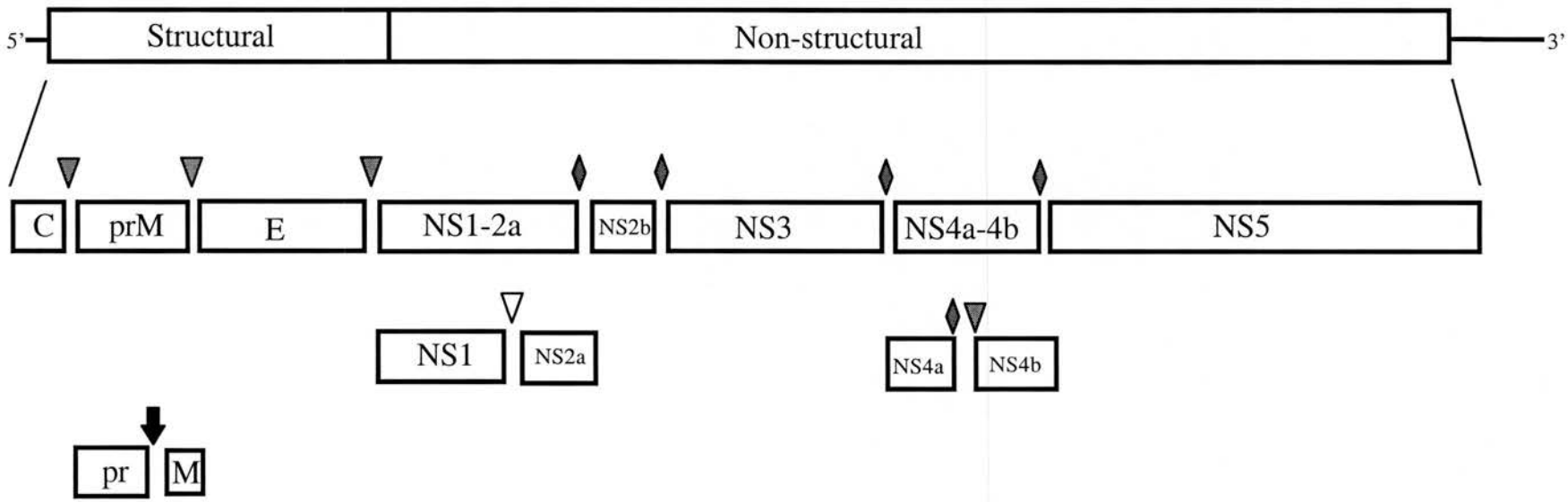
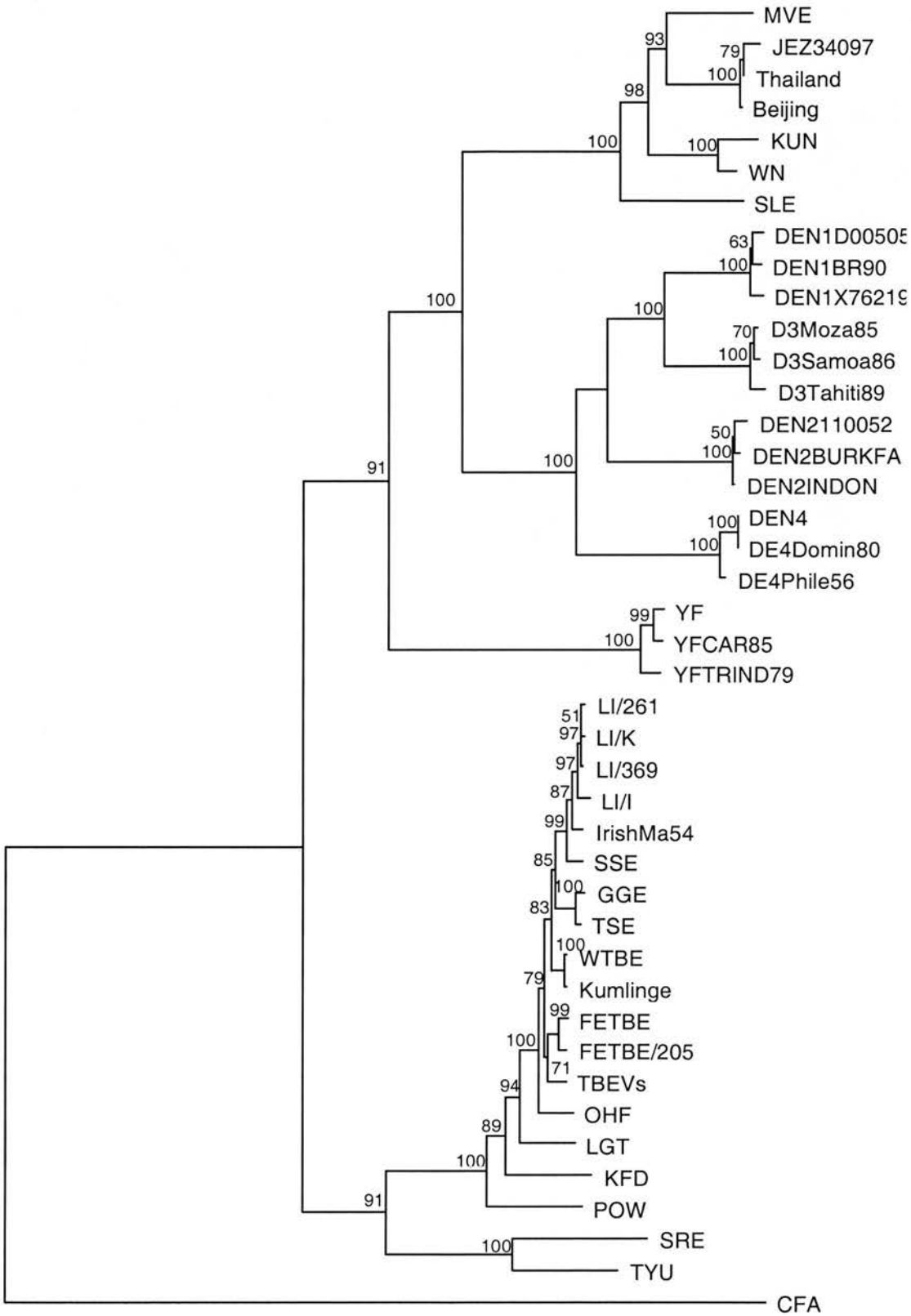


Fig. 1.1. Diagram illustrating flavivirus polyprotein processing. ▼ denotes signal peptidase catalyzed cleavage and ▽ denotes the involvement of an unknown protease. Both enzymes act in the lumen of the endoplasmic reticulum. ◆ denotes the action of the NS2b-NS3 protease within the cytosol and ➡ denotes the late cleavage of prM to M by an unidentified protease. Adapted from Chambers *et al.* (1990a).

Fig. 1.2. Neighbour-joining phylogenetic tree illustrating the evolutionary relationships of viruses in the genus *Flavivirus* (Gould *et al.*, 1997). The tree was constructed using the 1st and 2nd codon positions for 41 E genes. All horizontal branch lengths are drawn to scale. Percentage bootstrap support values are shown above the branches. The root for the genus separating tick-borne and mosquito-borne groups was determined based on the use of CFA virus as an outgroup.

Fig. 1.2.


MVE
JEZ34097
Thailand
Beijing
KUN
WN
SLE
DEN1D00505
DEN1BR90
DEN1X76219
D3Moza85
D3Samoa86
D3Tahiti89
DEN2110052
DEN2BURKFA
DEN2INDON
DEN4
DE4Domin80
DE4Phile56
YF
YFCAR85
YFTRIND79
LI/261
LI/K
LI/369
LI/I
IrishMa54
SSE
GGE
TSE
WTBE
Kumlinge
FETBE
FETBE/205
TBEVs
OHF
LGT
KFD
POW
SRE
TYU
CFA
93
79
100
98
100
100
63
100
100
70
100
50
100
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100
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91
100

CHAPTER TWO

Materials and Methods

CONTENTS

2.1. Viruses

Forty three viruses identified antigenically as LI virus, which were collected between 1931-1995, were included in this study. Information on their geographical origin, the year of isolation and the host species from which they were recovered is summarized in Table 2.1 and Fig. 2.1. The majority of locations correspond to the Veterinary Investigation (VI) centre from where the viruses were obtained by the Moredun Research Institute. HUR1 virus was supplied by Dr. J. S. Porterfield of the Sir William Dunn School of Pathology, Oxford.

Antigenic analysis has been carried out on three of the viruses during previous studies investigating LI virus variation. BRIS1, IRE1 and IRE2 virus were investigated by Hussain (1990) and Hubalek *et al.* (1995) under the designations LI/1065, MA14 and MR46 respectively. BRIS1 and IRE2 virus were also included in the study by Gao (1995).

The complete E gene sequences of eleven LI viruses have previously been determined (Shiu *et al.*, 1991; Venugopal *et al.*, 1992; Gao *et al.*, 1993b; Gao, 1995) and included in the phylogenetic analysis to allow the fullest investigation of LI virus variation possible. These included LI/31, the prototype strain of LI virus. Table 2.2 summarizes details pertaining to the isolation of these viruses as well as that of closely related flaviviruses which were also included in the phylogenetic analysis.

The passage history is known for all 43 viruses included in this study. With the exception of INV4 virus, all viruses were passaged twice in suckling mice. INV4 virus was cultured once in PS cells, between the two passages in suckling mice. No attempt was made to plaque purify the viruses and stocks were prepared as 10% (w/v) suspensions of infected suckling mouse brain (SMB) in phosphate buffered saline (PBS) and stored at -70°C.

2.2. Chemical and Biological Reagents

All chemicals and biological reagents used in this study are commercially available, and supplied by the following companies; Aldrich Chemical Company, Amersham International, Bioline (UK), British Drug House (BDH), Dynal (UK) Ltd., Gibco BRL, Iowa Biotechnology Corporation, New England Biolabs, Promega Corporation, Sigma Chemical Company and United States Biochemical (USB).

The radiochemicals used for the sequencing reactions were purchased from Amersham International as [α-^{35} S] dATP [600 Ci/mmol; 10 μCi/μl]

Solutions used in the course of this study were sterilized either by autoclaving or by filtration. To eliminate or reduce the potential problem of contamination while working with more than 40 closely related viruses, the following precautions were taken. Viruses were worked on sequentially. All reagents and primers used during the molecular analysis of LI viruses were dispensed into Eppendorf tubes which contained enough reagent for one reaction, or the tube was stored and only used again during work with the same virus. All primers and reagents were dispensed in an environment where contamination by LI virus was unlikely, i.e. in another laboratory in the building.

2.3. Pig Kidney Cell Lines

2.3.1. Pig Kidney Stable (PS) Cell Line

Pig kidney stable (PS) cells, obtained from the Institute of Poliomyelitis and Viral Encephalitides, Moscow, were used during the indirect immunofluorescence analysis. Cells were cultured in 150 cm^2 culture flasks in Leibovitz medium L15 with 5% foetal calf serum (FCS) and incubated at 37°C in a 5% CO_2 environment. When confluent, cells were detached with a 0.05% (w/v) trypsin and 0.02% (w/v) ethlyenediamine-tetra-acetate (EDTA) solution. The cells were resuspended in fresh L15 medium containing 5% FCS and passaged at an expansion rate of 1:3.

To ensure a constant supply of cells at a similar passage level a stock of PS cells was prepared. When confluent, the cells were detached in the manner described above and resuspended in L15 medium with 50% FCS and 10% dimethyl sulphoxide (DMSO). The cell suspension was dispensed in 1 ml quantities and stored in liquid nitrogen until required.

2.3.2. Pig Kidney IB/RS2 Cell Line

The pig kidney (PK) IB/RS2 clone 60 cell line from the Institute of Animal Health, Pirbright was used for the plaque assay analysis. A stock of PK cells was prepared in the same manner as described for PS cells and all experimental work was performed using cells between the 86th and 95th passage level. The PK cells were cultured at 37°C in a 5% CO_2 incubator. Passage of the cell line occurred every three to four days once a confluent monolayer had formed in 150 cm^2 culture flasks. The cell monolayer was washed in PBS at pH 7.2 before being detached with a 0.1% (w/v) trypsin and 0.05% (w/v) versene solution. The cells were resuspended in medium 199 with 10% newborn calf serum (NBCS) and passaged at an expansion rate of 1:3.

2.4. Plaque Assay

The morphology of plaques produced by LI viruses was investigated. Monolayers of PK cells in 12-well flat-bottomed tissue culture plates were used for plaque analysis and neutralization tests. PK cells were detached from 150 cm^2 tissue culture flasks as described above (Section 2.3.2). The cells were centrifuged at 500 g for 10 minutes and resuspended in fresh 199 growth medium. A single cell suspension was obtained by repeatedly drawing the cell suspension through a 26 gauge needle. A cell count was performed and the cell suspension adjusted to 3 x 10^5 cells per ml.

Two ml of the cell suspension was then aliquoted into each well of the 12-well plates. The plates were then incubated overnight at 37°C in a CO_2 incubator, by which time a confluent monolayer had formed.

Four serial ten-fold dilutions of each virus were made in PK/BA medium (Appendix 2.1). Medium 199 was removed from the PK cell monolayer and 0.2 ml of each virus dilution was inoculated into three wells. Plates were incubated at 37°C for 2 hours, during which time they were frequently rocked to distribute the inoculum evenly over the cells and prevent desiccation. After this adsorption period 2 ml carboxymethylcellulose (CMC) overlay medium (Appendix 2.2) was added to each well and the plates were incubated for 90-100 hours.

After the incubation period 0.5 ml 10% (v/v) formal saline was added to each well to inactivate the virus and fix the cells. After 40 minutes the overlay medium and formol saline were removed and the cell monolayer was washed three times with PBS. The monolayer was then stained with 0.1% (w/v) crystal violet in 20% (v/v) ethanol in distilled water. Plaques were counted and expressed as the mean number of plaque forming units (p.f.u.) per 0.2 ml. The plaque size was estimated using light microscopy by superimposing a grid of 0.25 mm x 0.25 mm squares onto the plaque and counting the number of squares containing disrupted cells. The average plaque size and standard error were determined by measuring the area of ten plaques for each virus investigated.

2.5. Monoclonal antibodies

The panel of monoclonal antibodies (MAbs) used for the indirect immunofluorescence analysis included 14 MAbs raised against the prototype LI virus LI/31, which had been previously prepared and characterized (Hussain, 1990; Venugopal *et al.*, 1992). The MAbs 3.1, 3.3, 4.2 and 7.1 are E protein-specific MAbs, while MAbs 1.1, 1.2, 3.2, 4.1, 7.3, 7.4, 7.5, 7.6, 8.1 and 8.2 are NS1 protein-specific MAbs. The E protein-specific MAb F7/3, which is broadly cross-reactive amongst the flaviviruses, was used as a positive control. The E protein-specific MAb

86.25 was used as a negative control as it recognizes a unique epitope of YF virus (Gould *et al.*, 1990).

All MAbs were employed as ascitic fluid preparations diluted in PBS. To obtain the optimum reaction conditions against viral antigens, MAbs 1.2, 3.2 and 8.2 were diluted 1:1000, MAb 7.4 was diluted 1:1500 and the remaining MAbs were diluted 1:500. These optimal dilutions had been determined previously in titration experiments.

2.6. Indirect Immunofluorescence Analysis

Antigenic variation among LI viruses was investigated by indirect immunofluorescence (IIF) analysis using the MAbs described above (section 2.5). PS cells were detached from flasks in the manner described in section 2.3.1 and resuspended in L15 medium. The virus preparation was added to the cell suspension at a predetermined dilution and incubated at room temperature for 40 minutes. The cell and virus suspension was transferred to Petri dishes containing 13 mm diameter coverslips and incubated at 37°C for 48 hours by which time a confluent monolayer had formed and approximately 25% of cells contained viral antigens. The cells were washed in PBS before being fixed for 10 minutes in 100% acetone and stored at -20° C until required.

Antibodies were added to the coverslip preparations at predetermined optimal dilutions for 40 minutes at 37°C. The cells were washed in PBS ensuring contamination by different antibodies could not occur. Anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate was then added for 40 minutes at 37°C. After a further wash in PBS the cells were mounted in 90% glycerol/saline containing the anti-fade agent Dabco and examined for fluorescence by ultraviolet microscopy.

2.7. Plaque Neutralization Tests.

Plaque neutralization tests were carried out using MAbs 4.2 and 7.1 to confirm the identification of escape variants made by IIF analysis. Cell monolayers in 12-well flat-bottomed plates were prepared in the manner described above (section 2.4). Virus suspensions were diluted in PK/BA medium to produce approximately 30-40 p.f.u. per 0.2 ml in control cultures. Ten-fold serial dilutions of MAbs 4.2 and 7.1 were made in PBS to which an equal volume of virus in PK/BA medium was added. The control experiment consisted of the virus dilution and PBS without antibody. The virus and antibody mixture was incubated at 37°C for 30 minutes. After this period 0.2 ml aliquots of the mixture was inoculated onto the PK cell monolayers. The plates were incubated at 37°C for 2 hours, during which time they were frequently rocked to distribute the inoculum evenly and prevent desiccation. After the incubation period, 2 ml CMC overlay medium was added to each well and then the plates were incubated at 37°C for 90-100 hours. The cells were then processed as described above in section 2.4.

The titres of specific neutralizing antibody activity were expressed as the reciprocal of the dilution of the antibody which reduced the plaque count by 75% (PRN_{75}). This value was calculated using the equation:

$$PRN_{75} = (-\log_{10} a) - (\log_{10} b) + (\log_{10} c)$$

where **a** denotes the antibody dilution at which the plaques were counted; **b** denotes the mean number of plaques in the control divided by 4; and **c** denotes the number of plaques observed at the antibody dilution of **a**.

2.8. Extraction of RNA

Two methods for the extraction of viral RNA from the SMB suspensions were employed and compared. In the first method the SMB was denatured using guanidinium isothiocyanate before extraction and precipitation of RNA using phenol: chloroform and isopropanol respectively (Chomczynski & Sacchi, 1987). This process took approximately four hours while the second method, using the surfactant Catrimox-14 (Iowa Biotechnology Corp.) required about one hour, involved fewer steps and resulted in similar quantities of RNA (Macfarlene & Dahle, 1993). Therefore the latter method was used for RNA extraction and is described in more detail below.

One ml Catrimox-14 was added to 100 µl SMB suspension and incubated at room temperature for 40 minutes. During this time Catrimox-14 forms a complex with RNA which can be precipitated by centrifugation for 5 minutes. The supernatant was discarded and the remaining pellet washed in water before the addition of 2M lithium chloride. Lithium chloride disassociates the complex and dissolves DNA and protein contaminants leaving a pellet of insoluble RNA after centrifugation for 5 minutes. This pellet is washed in 70% ethanol, dried, resuspended in water and stored at -20°C until required.

2.9. Synthesis of First Strand cDNA

The first strand of complementary DNA (cDNA) was synthesized from the extracted RNA using the downstream primers LIE2 (5'-GTAGTATGCATAGTT-3') and EXP2 (5'-ATCCGCTCCCCACCCGAGAGT-3') complementary to LI virus nucleotides 2449-2463 and 2341-2361 respectively (Shiu *et al.*, 1991). Initially the primer and extracted RNA were incubated at 95°C for two minutes to allow annealing of the primers before the mixture was cooled on ice. Reverse transcription was carried out by Superscript II murine reverse transcriptase enzyme (Gibco BRL) during 2 hours

incubation at 42°C (Gritsun & Gould, 1995). The reaction was terminated by incubation at 70°C for 10 minutes and the cDNA was stored at 4°C until required.

2.10. Amplification of the Envelope Gene.

The E gene was divided into six regions of between 250-500 nucleotides which were of a suitable length for direct solid-phase sequencing. For each region two sets of primers were designed, one set for the polymerase chain reaction (PCR) amplification of the region and the second set for the sequencing reaction. Different primers were used in the PCR and sequencing reactions to prevent the sequencing of non-specific PCR products. Information pertaining to the regions and primers is summarized in Table 2.3.

The final volume of the PCR mixture was 100 µl which contained cDNA, 30 pmol of each primer, 20 mM Tris-HCl (pH 8.3 at 20°C), 50 mM potassium chloride, 0.1% Tween-20, 2 mM magnesium chloride, 200 µM of each deoxynucleoside triphosphate (dNTP) and 1-2 units of *Taq* polymerase (Bioline, UK). The solution was overlaid with paraffin oil. A typical cycle used for the PCR was as follows, 95° C for 40 seconds, one minute at the annealing temperature, followed by 1.5 minutes at 72°C. The annealing temperature varied depending on the primers involved. The thirtieth cycle, the termination cycle, ended with 20 minutes at 72°C. All PCR reactions were carried out on a Hybaid thermocycler.

2.11. Sequencing of the Envelope Gene

The amplified regions of the E gene were sequenced by direct solid-phase sequencing using Streptavidin Dynabeads (Dynal UK Ltd.). This allowed purification of the PCR product and easy separation of the two strands of DNA before sequencing (Hultman *et al.*, 1989). The protocols used are described in the Dynal product manual and summarized below.

One of the primers used for PCR amplification was biotinylated and therefore capable of binding to Streptavidin Dynabeads under suitable conditions, i.e. 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 2 M sodium chloride. Once attached to the Dynabeads the DNA was washed and purified. After incubation for 10 minutes in 0.1 M sodium hydroxide the two strands of DNA separate so that one is still attached to the Dynabeads and the other is released into the supernatant medium thus enabling separation of the DNA strands.

Thereafter the DNA strands were sequenced using the USB Sequenase II sequencing kit as described in the protocol manual. The addition of stop solution at the end of the reaction resulted in the separation of the DNA from the Dynabeads to facilitate normal migration of the DNA on the sequencing gel.

2.12. Phylogenetic Analysis of Data

Phylogenetic analysis was initially carried out on region three (see Table 2.3) which contained two of the genetic markers; the tick-borne specific marker (Shiu *et al.*, 1991) and the species-specific marker (Shiu *et al.*, 1992a). In addition, the complete E gene was determined for nine LI viruses and phylogenetic analysis was carried out on these viruses and other LI viruses and closely related flaviviruses which had previously been investigated (Table 2.2).

Multiple and pairwise nucleotide and amino acid sequence alignments were constructed using the University of Wisconsin Genetics Computer Group (GCG) package. Phylogenetic trees were kindly reconstructed by Dr. E. Holmes (University of Oxford) using the maximum likelihood method (PAUP, version 4.54d, D.L. Swofford, Smithsonian Institution, Washington D.C., 1997). Bootstrap resampling (1000 replications) was used to assess the robustness of the groupings obtained.

2.13. Estimation of Rates of Substitution & Lineage Divergence Times.

Approximate dates of isolation are available for all viruses included in this study and therefore it is possible to estimate the rate of synonymous substitutions. The nonsynonymous substitution rate was not investigated due to the high sequence similarity of these viruses. The synonymous pairwise genetic distances (d_s) between sequences were first generated using the Nei-Gojobori (1986) method available in the MEGA software package (Kumar *et al.*, 1993). Multiple substitutions and back mutations may have occurred in these sequences which would not be accounted for in the calculated d_s values and would lead to an underestimation of the substitution rate. To overcome this the Jukes-Cantor formula, available in the MEGA software package, was used to correct the d_s values.

$$\text{Jukes-Cantor correction} = -3/4 \ln (1-4/3d_s)$$

The model for calculating the rate of synonymous substitution is illustrated in Fig. 2.2 (Li *et al.*, 1988).

By assuming a constant rate of substitution prior to isolation and that no nucleotide substitutions have occurred since isolation, the times when virus lineages diverged can be estimated using a model proposed by Li *et al.* (1988). The date of lineage divergence is calculated in the following manner. A derivation of the rate equation is:

$$t = d_s/a$$

where **t** denotes the time (in years) between the isolation of the sequences in question and **a** denotes the rate of synonymous substitution. Since d_s is the sum of the genetic distance of both sequences from the common ancestor (Fig. 2.2, l_1 and l_2) then:

$$t_1 + t_2 = d_s/a$$

where $\mathbf{t_1}$ and $\mathbf{t_2}$ are the dates when the viruses were isolated. If the time difference between $\mathbf{t_1}$ and $\mathbf{t_2}$ is **x** then:

$$2t_1 = d_s/a - x$$

and therefore the time of divergence ($\mathbf{t_0}$) will be:

$$t_0 = t_1 - [(d_s/a) - x]/2$$

The average rate of synonymous substitution calculated in this study was used to estimate lineage divergence times for LI viruses and closely related flaviviruses.

2.14 *In vivo* Studies

2.14.1 Pathogenicity Studies

Two strains of mice were used during the course of the project. Initially, the virulence characteristics of ten LI viruses was investigated in 3-5 week old inbred C57 black mice (C57/BL) (strain 6J) bred at the Moredun Research Institute. The mice were divided into groups of 20 containing equal numbers of male and female animals. The viruses under investigation were diluted in PK/BA medium so that each mouse received between 1.2-3.4 x 10^3 p.f.u. of virus in 0.2 ml inoculum. The viruses were inoculated by subcutaneous injection to mimic a tick-bite.

The mice were observed for 21 days and euthanized, by the intraperitoneal inoculation of pentobarbitone, at the first clinical sign of disease. However, several mice died between the 12 hourly observations. The brains of all mice involved in the study were collected and prepared for histopathological studies. After 21 days the remaining mice were euthanized and blood for serum separation was collected and tested for HI antibody, kindly performed by Miss I. Pow (Moredun Research Institute).

The results of the study were inconclusive and therefore the virulence characteristics of three LI viruses were investigated further in 3-5 week old random bred Swiss white (SW) mice, bred at the Moredun Research Institute, using the same protocol described above. Each mouse received between 1.9-2.1 x 10^3 p.f.u. of virus by subcutaneous inoculation.

2.14.2 Investigation of Virus Titres in the Blood and Brain

The virulence characteristics of the ten viruses analyzed in section 2.14.1 was studied further with the investigation of the titres of virus in the brain and blood of C57/BL mice during the first 7 days of infection. The mice, aged 5-7 weeks, were divided into groups of 20 containing equal numbers of male and female animals. The viruses under investigation were diluted in PK/BA medium so that each mouse received 0.2 ml inoculum containing between 1.7-8.3 x 10^2 p.f.u. of virus. The viruses were inoculated by subcutaneous injection.

On days 1, 3, 5, and 7 five mice from each group were euthanized by the intraperitoneal inoculation of pentobarbitone. 0.2 ml blood samples were collected from each mouse and the brains were removed and stored at -70°C for later analysis.

Viraemia titres were investigated by plaque assay. The blood samples were diluted 1:10 (v/v) in PK/BA medium. After centrifugation at 1500 g for 10 minutes the supernatant was analyzed using the protocol described in section 2.4. Virus titres in the brain were also investigated by plaque assay. Each brain was weighed and a 1:10 (w/v) dilution was made in PK/BA medium. The brain suspension was homogenized and centrifuged at 1500 g for 10 minutes and the supernatant was used for plaque assay analysis, as described in section 2.4.

A comparison of virus titres present in the brains of C57/BL and SW mice was made for three LI viruses. SW mice, aged 3-5 weeks, were inoculated subcutaneously with between 1.9-2.1 x 10^3 p.f.u. of virus. The experiment terminated on day six instead of day 7 due to the earlier onset of disease observed in SW mice compared to C57/BL mice.

2.15. Haemagglutination-Inhibition Analysis

To confirm that infection resulted from the subcutaneous injection of all mice which survived virus challenge, blood samples were taken at the end of the experiment and analyzed for HI antibody. The basic protocol is adapted from Clarke & Casals (1958). Ten percent dilutions of 100 µl serum samples were made in 0.5% bovine albumin in borate saline. To remove non-specific inhibitors of haemagglutination the sample was treated for a least 20 minutes with an equal volume of a 25% (w/v) suspension of acid washed kaolin. The kaolin was sedimented by centrifugation and the supernatant treated with 50 µl packed gander erythrocytes for 20 minutes at room temperature to remove non-specific agglutinins. After centrifugation the supernatant of each sample is equivalent to a 1:20 dilution of the original sample.

Two fold dilutions of each sample were made to give a final volume of 0.2 ml in each well of a standard 96 well plate. An equal volume of LI virus heamagglutinin antigen was added to each well and incubated overnight at 4°C. After this period 0.4 ml of 0.25% gander erythrocytes in adjusting diluent, giving a final pH of 6.2-6.4, were added to each well before incubation at room temperature for one hour until the erythrocytes in the control wells, which contain only the antigen, had settled. The highest dilution where heamagglutination was completely inhibited was taken as the end point dilution.

2.16. Histological Examination

The brains of all clinically affected mice were harvested and fixed in 1% Bakers saline for at least 72 hours. In addition the brains of several control animals and mice which survived challenge were also harvested. Coronal sections, each of approximately 3 mm in width, were made of the brains before processing through paraffin wax. Five µm sections of the brain were cut and stained with heamatoxylin and eosin (H&E) before examination by light microscopy.

2.17. Statistical Analysis

Where applicable, standard statistical analysis was carried out through the calculation of means and standard errors. Associations between variables detected in the *in vivo* studies were investigated using the Spearman rank correlation coefficient.

Further analysis was kindly carried out by Dr. I. McKendrick (BioSS). Plaque morphology data was log transformed and tested by one-way analysis of variance. The size of plaques produced by different viruses was compared using t-test analysis using the pooled standard deviation derived from the analysis of variance. The associations between plaque morphology and geographic location and antigenic phenotype were investigated using REML, an extension of the analysis of variance which will cope with unbalanced data sets. The significance of the effects which were estimated was tested using the Wald test, which is asymptotically chi-squared.

Differences in the cumulative mortalities of mice caused by the inoculation of different LI viruses were tested using estimates derived from a profile likelihood model (Clayton & Hills, 1993) and tested using the fact that these statistics are well approximated by the Normal distribution. To compare the cumulative mortalities of C57/BL and SW mice the day of death of each mouse was ranked and tested by one-way analysis of variance.

Virus	Year	Host Species	Location
BRIS1	1985	ovine	Bristol
DEV1	1983	ovine	Devon
DEV2	1989	ovine	Devon
DEV3	1989	ovine	Devon
DEV4	1995	ovine	Devon
HUR1	1931	ovine	Scotland
INV1	1983	ovine	Inverness
INV2	1983	ovine	Inverness
INV3	1983	ovine	Inverness
INV4	1985	ovine	Inverness
INV5	1986	ovine	Inverness
INV6	1986	ovine	Inverness
INV7	1987	ovine	Inverness
INV8	1987	ovine	Inverness
INV9	1987	ovine	Inverness
INV10	1987	ovine	Inverness
INV11	1988	ovine	Inverness
INV12	1988	ovine	Inverness
INV13	1988	ovine	Inverness
INV14	1992	ovine	Inverness
INV15	1993	porcine	Inverness
IRE1	1967	ovine	Dublin
IRE2	1971	tick	Dublin
IRE3	1968	ovine	Dublin
IRE4	1972	tick	Dublin
LOCH1	1993	grouse	Lochindorb
LOCH2	1993	grouse	Lochindorb
LOCH3	1993	grouse	Lochindorb
LOCH4	1993	grouse	Lochindorb
LOCH5	1993	grouse	Lochindorb
LOCH6	1993	grouse	Lochindorb
OBAN1	1982	ovine	Oban
PEN1	1983	ovine	Penrith
PEN2	1983	ovine	Penrith
PEN3	1983	ovine	Penrith
PEN4	1983	ovine	Penrith
PEN5	1983	ovine	Penrith
PEN6	1983	ovine	Penrith
PRES1	1991	ovine	Preston
THK1	1986	grouse	Thirsk
THO1	1984	ovine	Thurso
THO2	1990	ovine	Thurso
WEST1	1993	ovine	Westerdale

Table 2.1. Alphabetically arranged list of 43 LI viruses included in this study. This table contains information on the; year, host species and the general location from which the viruses were isolated. Lochindorb is an estate on Speyside.

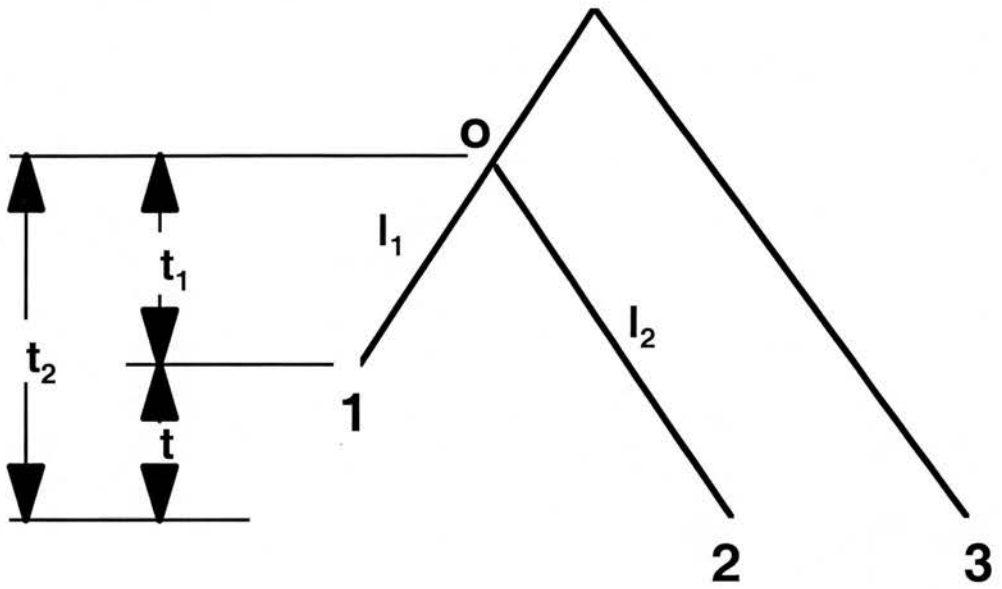
Virus	Designation	Geographic Origin	Year of Isolation	Host Species	Source of Sequence Data	Accession Number
GGE		Greece	1969	caprine	Marin *et al.* (1995a)	X77732
FETBE	Oshima	Japan	1993	human	Takashima *et al.* (1997)	AB001026
FETBE	Sofjin	Russia	1937	human	Yamshchikov & Pletnev (1988)	X07755
KFD		India	ND	ND	Venugopal *et al.* (1994a)	X74111
LGT	TP21	Malaysia	1956	tick	Mandl *et al.* (1991a)	M73835
LI	LI/31	Scotland	1931	ovine	Gao *et al.* (1993b)	D12937
LI	LI/261	Newcastle	1987	ovine	Gao (1995)	X86787
LI	LI/369	Ayrshire	1963	tick	Shiu *et al.* (1991)	M37687
LI	LI/917	Penrith	1985	ovine	Gao (1995)	X86786
LI	LI/A	Devon	1980	ovine	Gao (1995)	X69975
LI	LI/G	Mull	1979	porcine	Gao (1995)	X86788
LI	LI/I	Aberystwyth	1980	ovine	Gao (1995)	X86785
LI	LI/K	Grampians	1980	grouse	Gao *et al.* (1993b)	D12935
LI	LI/NOR	Norway	1984	ovine	Gao *et al.* (1993b)	D12936
LI	MA54	Ireland	1968	bovine	Gao (1995)	X86784
LI	SB/526	Oban	1968	ovine	Venugopal *et al.* (1992)	M94957
OHF	Bogolubovka	Russia	1964	human	Gritsun *et al.* (1993b)	X66694
POW	LB	Canada	1958	human	Mandl *et al.* (1993)	L06436
SSE		Spain	1987	ovine	Marin *et al.* (1995a)	X77470
TBE	Vasilchenko (Vs)	Russia	1969	human	Gritsun *et al.* (1993a)	M97369
TSE		Turkey	1969	ovine	Gao *et al.* (1993a)	X69125
WTBE	263	Czech Republic	1987	tick	Wallner *et al.* (1996)	U27491
WTBE	4387	Slovakia	1982	bank vole	Labuda *et al.* (1994)	X76607
WTBE	Kumlinge-A52	Finland	1959	tick	Whitby *et al.* (1993a)	X60286
WTBE	Hypr	Czech Republic	1953	human	Wallner *et al.* (1996)	U39292
WTBE	Neudoerfl	Austria	1971	tick	Mandl *et al.* (1988)	M27157

Table 2.2. Table summarizing information on the additional LI viruses and closely related TBE serocomplex viruses studied in this investigation. ND indicates no data were available.

Region	PCR Primers		Sequencing Primers		Fragment Size
	Upstream	Downstream	Upstream	Downstream	(nucleotides)
1	EXP1 781-801	MLE6* 1116-1135	LIEIN 805-821	MLE4 1087-1107	266
2	MLE7* 953-971	MA2 1441-1460	MA1 992-1008	MA2 1441-1460	470
3	EXTI* 1295-1314	EXT2 1746-1767	INT1 1375-1394	INT2 1717-1736	322
4	INT1 1375-1394	MLE1* 2359-2380	LIE3 1609-1626	LIE4 1982-1999	366
5	LIE3 1609-1626	MLE1* 2359-2380	MLE5 1870-1890	SEQ2 2182-2201	291
6	MLE9* 1984-2003	LIE2 2449-2463	MLE3 2052-2072	LIE2 2449-2463	376

Table 2.3. Table summarizing information concerning the primers used for sequencing the E gene. The symbol * indicates the biotinylated primers. Positions indicated are relative to the sequence of the 5' portion of the genome of LI/369 (Shiu *et al.*, 1991).

Fig. 2.1. Map of the British Isles indicating the source of LI viruses included in this study. Lochindorb in an estate on Speyside.



Equation for calculating the rate of synonymous substitution

$$l_2 - l_1 = at_2 - at_1 = at$$

by using the outgroup sequence 3 as a reference:

$$l_2 - l_1 = d_{23} - d_{13} = at$$

therefore

$$a = (d_{23} - d_{13})/t$$

Fig 2.2. Model for estimating the rate at which nucleotide substitutions occur (Li *et al.*, 1988). Numbers **1-3** denote related virus sequences, **o** denotes the ancestral node, $\mathbf{t_i}$ denotes time of isolation of sequence i (in years), $\mathbf{l_i}$ denotes the expected number of substitutions/site for sequence i from the ancestral node of the sequence to the time of isolation, **a** denotes the number of substitutions/site/year and $\mathbf{d_{ij}}$ denotes the number of substitutions/site (genetic distance) between sequences i and j.

CHAPTER THREE

Antigenic Variation Among Louping-ill Viruses

CONTENTS

3.1. Introduction

Compared with other RNA viruses tick-borne members of the genus *Flavivirus* exhibit extraordinary antigenic stability (Stephenson *et al.,* 1984; Guirakhoo *et al.,* 1987; Heinz *et al.,* 1990). For example, WTBE viruses from Finland and Austria are virtually indistinguishable by MAb analysis. LI virus is no exception and 21 LI viruses from across Britain could not be distinguished by neutralization using polyclonal antisera, by haemagglutination activities nor by plaque morphology and mouse pathogenicity studies (Reid, 1984). However, variation was detected in a later study involving the comparison of 18 viruses using MAbs (Hussain, 1990). In particular the study identified a group of viruses which failed to bind to, or be neutralized by, two E protein-specific MAbs, 4.2 and 7.1 (Hussain, 1990; Gao *et al.*, 1994). These MAbs were the only neutralizing MAbs generated in the study and in addition MAb 7.1 exhibited HI activity. The viruses which were not recognized by MAbs 4.2 and 7.1 can therefore be described as escape variants. However, this antigenic characteristic has not been associated with immune selection pressures and therefore the term 'escape variant' may be inappropriate. Nevertheless, viruses with this antigenic phenotype have previously been described as escape variants (Jiang *et al.*, 1993; Gao *et al.,* 1994) and therefore this terminology has been used throughout this thesis. LI virus escape variants are the only naturally occurring escape variants identified for a flavivirus species (Gao *et al.,* 1994).

Viruses with the same antigenic characteristics were selected under experimental conditions in the laboratory by culturing the virus in the presence of MAb 4.2 (Jiang *et al.*, 1993). It was noted that the MAb 4.2 escape variant phenotype always accompanied the MAb 7.1 escape variant phenotype implying that the MAbs recognize epitopes which are either closely situated to one another or partially overlapping. The MAbs are unlikely to recognize the same epitope due to their differing biological activities. A similar situation was reported for WTBE virus escape variants (Holzmann *et al.*, 1989, 1990). Experimentally recovered escape variants selected in the presence of MAb B1 were not recognized by this MAb or

MAb B4. In addition, both MAbs failed to bind to escape variants selected in the presence of MAb B4.

The importance of escape variants in the epizootiology of LI virus is unknown. Naturally occurring escape variants have only been identified in England and Wales and not from Ireland or Scotland, where the majority of samples have been isolated. This may be due to differences in farm practices, particularly with respect to vaccine usage. Naturally occurring escape variants of infectious bursal disease (IBDV) virus (Family *Birnaviridae*) have only been isolated in areas of America where intensive poultry farming occurs and where extensive use of the available vaccine has been made (Snyder *et al.*, 1992). In addition, all IBDV virus escape variants identified in the study were collected after 1985. Similarly, LI virus escape variants have only been isolated since 1980, although all earlier viruses were collected in Scotland and Ireland and therefore it is unclear whether the temporal or spatial elements are important in the distribution of escape variants.

Escape variants have been generated in the laboratory for a number of flaviviruses including; TBE virus (Holzmann *et al.*, 1990, 1997), JE virus (Cecilia & Gould, 1991), YF virus (Lobigs *et al.*, 1987; Sil *et al.*, 1992), MVE virus (McMinn *et al.*, 1995a) and DEN virus (Lin *et al.*, 1994; Hiramatsu *et al.*, 1996). Most of these escape variants were the result of single amino acid substitutions, and this was found to be the case for LI virus. Single amino acid substitutions at residues 308, 310 or 311 within the E protein are responsible for both naturally occurring and experimentally selected escape variants (Jiang *et al.*, 1993; Gao *et al.*, 1994). These residues lie within the putative receptor binding domain in the TBE virus E protein according to the three dimensional structural model (Rey *et al.*, 1995). The substitution of Asp-308 to Asn was found to cause the escape variant characteristic in a number of experimentally selected escape variants. This substitution has not been found in naturally occurring escape variants, although the substitution Asp-308 to Glu was found in LI/A, a virus isolated in Devon. The remaining naturally occurring escape variants have substitutions at residue 311; LI/I virus encodes the substitution Lys-311 to Gln while LI/261 and LI/917 virus have the substitution Lys-311 to Arg.

Experimentally selected escape variants were found to encode the substitutions Lys-311 to Gln or Asn as well as Ser-310 to Pro.

Passage of DEN virus in mice or cell lines selects for particular amino acid substitutions in the E protein which are situated near the hinge-like region of domain II in the three dimensional protein E model (Rey *et al.,* 1995; Lee *et al.,* 1997). Therefore it is possible that mutations in residues 308-311, which lie within the IgC-like module of the E protein, may also be an artefact of serial passage in the laboratory. However, in the study by Hussain (1990) identical procedures were used for all viruses and only four out of 18 exhibited the escape variant phenotype. In addition, WTBE virus escape variants can be selected in the presence of MAb 4.2, although escape variants of this type have never been reported (Jiang *et al.,* 1993).

All LI virus escape variants were isolated from the CNS of diseased sheep which showed the typical clinical symptoms of LI virus infection. However, the escape variants tend to exhibit smaller plaque sizes and lower virulence characteristics in mice after subcutaneous or intraperitoneal inoculation (Hussain, 1990; Jiang *et al.,* 1993; Gao *et al.,* 1994). This altered pathogenicity correlates with the amino acid substitution responsible for the escape variant characteristic (Jiang *et al.,* 1993). Experimentally selected escape variants with substitutions at residue 311 exhibit similar mouse virulence characteristics to the parent virus. Substitutions at residue 310 result in partial attenuation while the substitution of Asp-308 to Asn resulted in complete attenuation of the virus which failed to elicit an immune response implying that the virus was incapable of replicating in mice. A similar study was carried out on naturally occurring escape variants (Gao *et al.,* 1994). LI/I virus, with the substitution Lys-311 to Gln, was the most attenuated virus unlike the experimentally selected variant with the same mutation. This reflects the importance of other substitutions in the viral genome on the virulence characteristics of LI virus. LI/A virus, with the substitution Asp-308 to Glu, was attenuated but did replicate in mice and caused mortality in a proportion of the animals unlike the experimentally selected escape variant with the substitution Asp-308 to Asn. Therefore the presence of Asp, Glu or Asn at residue 308 has a profound effect on the virulence and biology of the virus.

In this study the antigenic variation among more than forty LI viruses was investigated using MAbs previously prepared against the prototype virus LI/31 (Hussain, 1990). Little antigenic variation was detected among the viruses. However, eight antigenic variants were identified which fall into two categories and the amino acid substitutions which induce the antigenic phenotypes were investigated. The biological characteristics of these viruses have also been compared to those of other LI viruses.

3.2. Results

3.2.1. Antigenic Analysis of LI viruses by IIF Analysis using E and NS1 Protein-Specific MAbs

Antigenic variation among LI viruses was investigated using the panel of MAbs described in section 2.5. The binding properties of the MAbs were investigated by IIF analysis and the typical fluorescence observed in virus antigen containing cells is illustrated in Fig. 3.1.

The results summarized in Table 3.1 clearly show that LI viruses exhibit little antigenic variation among the epitopes investigated. Differences were not detected in the binding properties of MAbs specific to the NS1 protein. The only variation observed was seen in the E protein-specific MAbs 4.2 and 7.1 and the LI viruses can be divided into three groups on the basis of the binding properties of these MAbs. The majority of viruses were recognized by all the MAbs, including MAbs 4.2 and 7.1, and these viruses are henceforth referred to as wild-type viruses. MAbs 4.2 and 7.1 did not bind to the LI viruses INV6 and PRES1. Naturally arising escape variants of this type have previously been described (Hussain, 1990; Gao *et al.,* 1994) and also selected under experimental conditions in the laboratory (Jiang *et al.,* 1993). Therefore variants of this type are henceforth referred to as type-I escape variants. The LI viruses BRIS1, DEV1, DEV2, DEV3, DEV4, and INV1 were recognized by

MAb 4.2 but not by MAb 7.1. Escape variants of this type have not been identified previously and are henceforth referred to as type-II escape variants.

The identified type-I escape variants were collected in the north of England and Scotland. The type-II escape variants were also collected in Scotland and the south-west of England. An additional virus from Devon, LI/A, was previously investigated and identified as a type-I escape variant. However, upon repeating the antigenic analysis of LI/A the virus was consistently found to exhibit type-II escape variant characteristics.

3.2.2. Plaque Neutralization Analysis

IIF analysis identified two types of escape variant, while previous studies have only identified one type. In an attempt to validate the results of this study plaque neutralization tests were carried out using the neutralizing MAbs 4.2 and 7.1. A total of 10 viruses were analyzed; six type-II escape variants DEV1, DEV2, DEV3, DEV4, LI/A and INV1, two type-I escape variants INV6 and PRES1 and two wild-type viruses SB/526 and INV14. The results are summarized in Table 3.2 and recorded as the negative logarithm ($\log_{10}$) of the end-point dilution of MAb which reduced the plaque count by 75 percent.

The neutralizing activity of MAb 4.2 varied between the viruses and this was correlated with the antigenic variation described above. As a result the viruses could be divided into three groups. Unfortunately too few samples were available for the detection of statistically significant differences. MAb 4.2 neutralized the type-II escape variants (mean end-point dilution 3.78 ± 0.09) although not to such a high degree as SB/526 virus, with an end-point dilution of 4.77. The type-I escape variants, INV6 and PRES1 virus, were also neutralized by MAb 4.2, although only at a higher antibody concentration than neutralization of the type-II escape variants (mean end-point dilution 2.16 ± 0.40)

The MAb 7.1 preparation used in this study appeared to have less neutralizing activity than MAb 4.2 at the same dilution and as a result lower end-point dilutions

were recorded. However, differences between the neutralizing activity were detected and these fell into two categories, although again too few samples were available for statistical analysis. MAb 7.1 exhibited minimal neutralization of the type-I and type-II escape variants, the mean end-point dilution was 0.72 ± 0.04 compared with 1.39 ± 0.20 for the wild-type viruses SB/526 and INV14.

Therefore the classification of LI viruses identified by the binding activities of MAbs 4.2 and 7.1 is supported by the groupings determined by analysis of the functional activities of these MAbs. However, at this stage it is not entirely clear why MAb 4.2 neutralizes type-I escape variants while failing to bind to these viruses in IIF analyses.

3.2.3. LI Virus Virulence in C57/BL Mice.

The results of the pathogenicity studies will be described in more detail in chapter five. However, it is appropriate to include the virulence characteristics of the escape variants in this chapter. The cumulative mortality of C57/BL mice subcutaneously inoculated with the wild-type virus, SB/526, was compared with that caused by type-I escape variants, INV6 and PRES1, and type-II escape variants, LI/A, INV1 and DEV4 (Fig. 3.2).

The two type-I escape variants exhibited similar cumulative mortality rates in C57/BL mice, of 65-75%, which were also comparable to that caused by the wild-type virus. The pathogenicity characteristics of the type-II escape variants were much more diverse. LI/A virus was the most attenuated LI virus analyzed, causing mortality in 35% of inoculated mice. DEV4 virus exhibited similar virulence characteristics to the type-I escape variants, while INV1 virus was the most pathogenic virus analyzed causing 95% mortality in inoculated mice.

3.2.4. Genetic Determinants of Antigenic Phenotypes

Five LI virus escape variants have been identified in previous studies and the E gene sequences have been determined for four of these viruses (Gao *et al.,* 1994; Gao, 1995). However, the results of this study imply that LI/A and BRIS1 virus, previously identified as type-I escape variants, were incorrectly classified and are in fact type-II escape variants. Therefore the findings of previous papers must be reinterpreted. The causative amino acid substitutions in both type-I and type-II escape variants are situated at the same region, residues 308-311, of the E protein and therefore this fragment of the E gene was sequenced for all eight escape variants identified in this study. The gene fragment sequenced, region 4 in Table 2.3, encompasses nucleotides 756-1110 encoding amino acids 253-370. In addition, the complete E gene sequences were determined for two type-II escape variants, INV1 and DEV4. A multiple alignment of the appropriate sequences is illustrated in Fig. 3.3 and includes the relevant sequences of previously identified escape variants.

PRES1 virus, a type-I escape variant, contains the substitution Lys-311 to Arg which has previously been observed in LI/261 and LI/917, also from the north of England. INV6 virus, the other type-I escape variant identified in this study, contains two substitutions within the variable domain, Asp-308 to Glu and Lys-311 to Arg. Neither of these substitutions is unique but INV6 virus is the first escape variant reported to contain more than one substitution in the variable domain. All type-II escape variants have the same substitution as LI/A virus, which involves the substitution of Asp-308 to Glu.

3.3. Discussion

This study reaffirms the close antigenic relatedness of LI viruses from across the British Isles, including Ireland. Gao (1995) sequenced a virus from Ireland, MA54, which shares relatively low amino acid sequence similarity (95.4-96.4%) with other LI viruses. Additional LI viruses from Ireland included in this study are antigenically

indistinguishable from LI viruses from Britain and a number of these viruses are genetically similar to MA54 virus (chapter 4). The antigenic similarity of British and Irish viruses clearly illustrates the limitations of the use of MAbs to investigate variation among closely related viruses.

Variation was detected in the binding properties of MAbs 4.2 and 7.1 by IIF analysis, with the identification of two types of variants. Therefore this study supports previous work with the identification of naturally occurring antigenic variants and extends their known range of distribution to include Scotland.

Three of the LI viruses included in this study have been investigated previously using the same panel of MAbs and contradictory results have been reported in each case. In a previous study four LI viruses from Ireland failed to bind to the NS1 protein-specific MAb 8.2 (Hussain, 1990). However, two of these viruses, IRE1 and IRE2, were included in this study and produced bright fluorescence as did additional Irish LI viruses. Two previous studies reported the antigenic characteristics of BRIS1 virus by IIF analysis (Hussain, 1990; Hubalek *et al.,* 1995) and the conclusions contradict each other and the results of this study. Hussain (1990) identified BRIS1 virus as a type-I escape variant which failed to bind to MAbs 4.2 and 7.1. Hubalek *et al.* (1995) reported that BRIS1 virus was recognized by both MAbs 4.2 and 7.1 while in this study the virus was identified as a type-II escape variant. The antigenic analysis of BRIS1 virus was repeated using different passages of the virus including the original virus sample investigated by the previous authors. No variation in the binding characteristics of the MAbs were observed, including those of MAbs 4.2 and 7.1.

The reasons behind these contradictory results are unknown but presumably result from differences in the technique used to prepare the infected cells or MAbs. Similar protocols were used for the preparation of coverslips, although the previous studies had used PK instead of PS cells which were used in this study (Hussain, 1990; Hubalek *et al.,* 1995). However, the antigenic reanalysis of LI/A virus was carried out in PK cells and still produced contradictory results to the previous analyses. The most probable explanation is the use of sub-optimal dilutions of MAbs which can lead to weak positive fluorescence being mistaken for negatives due to high

background, non-specific fluorescence. It is important to note that the identification of all viruses from the south-west of England as type-II escape variants by IIF analysis is supported by the plaque neutralization analysis.

The results of this study imply that MAb 4.2 neutralizes type-I escape variants but fails to bind to them in IIF tests. The virus infected PS cells used in the IIF analyses were fixed in acetone which has been reported to affect the conformation of epitopes in several viruses, e.g. measles and canine distemper virus (Gould *et al.*, 1981). Acetone-sensitivity of membrane associated proteins probably reflects the involvement of lipids in the conformational arrangement of the epitopes. Since neutralization tests are carried out without acetone treatment, it is entirely feasible that a negative IIF result could be obtained even though a neutralization test gives a positive result. Interestingly, the epitope of MAb 7.1 is either closely situated to or overlaps the MAb 4.2 epitope but exhibits no presumed acetone-sensitivity and fails to neutralize type-I and type-II escape variants. Nevertheless the neutralization results reported in section 3.2.2 lead to the classification of the investigated viruses into three groups, which is in complete agreement with that obtained by IIF analyses.

The amino acid substitutions responsible for the escape variant characteristics have been elucidated. The naturally occurring type-I escape variants result from a substitution at residue 311 and the type-II escape variants encode a change at residue 308. The genetic determinant is correlated to the geographic distribution of the viruses. All escape variants from the north of England have the substitution Lys-311 to Arg. The Welsh escape variant has the substitution Lys-311 to Gln, while the Scottish type-I escape variant encodes two substitutions, Asp-308 to Glu and Lys-311 to Arg. Type-II escape variants have only been isolated in Scotland and the south-west of England and result from an identical substitution, Asp-308 to Glu.

It is interesting to note that although escape variants were generated in the laboratory with the substitution Ser-310 to Pro, naturally occurring escape variants with this substitution have not been isolated in nature. There is also a difference in the causative substitution at residue 311 in experimentally selected and naturally occurring escape variants. The substitution of Lys to Arg is only found in naturally occurring escape variants while the substitution to Asp has only been recorded in

experimentally selected escape variants. The reasons behind these differences are not clear, although variants with experimentally selected mutations may be able to survive *in vitro* but not in the natural system involving the different cellular environments of vertebrate and invertebrate hosts.

Furthermore, substitutions of Lys-309 have not been reported. This residue may be important in maintaining the structural integrity of domain III and is conserved in all tick-borne flaviviruses (Jiang *et al.,* 1993). However, site-directed mutagenesis of a WTBE virus infectious clone has recently generated a virus with a substitution at residue 309. It is important to note that the virus is only viable because a second substitution, Phe-322 to Tyr, occurred within domain III which appears to alleviate the detrimental effect of the substitution at residue 309 stabilizing the structure of this domain and therefore the protein (Mandl *et al.,* 1996).

The substitutions at residue 308 are of particular interest because of their apparent effect on the biology and antigenicity of the virus. The presence of Asp at residue 308 results in a wild-type virus which binds to both MAbs 4.2 and 7.1. The presence of Asn at this site results in a type-I escape variant which is not viable in the mouse model and therefore it is not surprising that it has never been isolated in the wild (Jiang *et al.,* 1993). The presence of Glu at residue 308 encodes for type-II escape variants which have much more variable pathogenic properties. Experimentally selected escape variants with this substitution were not generated because they were cultured in the presence of MAb 4.2 and type-II escape variants are still recognized by this MAb. It would be interesting to investigate escape variants selected in the presence of MAb 7.1 to determine if type-I or type-II escape variants were generated and which had the greatest fitness.

Substitutions at residues 308-311 alter the binding capacity of MAbs 4.2 and 7.1 but it is unclear if these residues lie within the epitope and therefore have a direct effect on MAb binding or if they are located nearby and alter the conformation of the epitopes. The variable domain is found on the exposed surface of domain III and therefore could form part of an epitope (Rey *et al.,* 1995). MAb 4.2 recognizes a linear epitope while MAb 7.1 recognizes a conformational epitope which presumably overlaps the linear epitope (Jiang *et al.,* 1993). The variation in binding by MAb 4.2

is dependent on the residue present at position 308, implying that this residue lies near the epitope and induces a conformational change thus affecting the binding properties of the MAbs. A similar situation has been described in escape variants of foot and mouth disease virus (Family *Picornaviridae*) (Parry *et al.,* 1990).

The variable domain is next to the Cys-307 residue which forms half of the disulphide bond vital for the antigenic stability of domain III (Winkler *et al.,* 1987b; Guirakhoo *et al.,* 1989). Changes in residues near the disulphide bond may place a strain on the bond due to size or charge differences and alter the overall structure of the domain and therefore of the MAb 4.2 and 7.1 epitopes. The epitopes of the other two E protein-specific MAbs included in the MAb panel have not been localized within the protein and may lie outside domain III and therefore are not similarly affected by strain on the disulphide bond. If this theory is correct it may explain why the escape variants are more attenuated the closer the altered residue is to Cys-307 (Jiang *et al.,* 1993). The presence of Asn at residue 308 in experimentally selected escape variants has a much greater effect on the biology of the virus than the substitution of Asp for Glu. This may be due to the change in charge associated with the first substitution and would support the above theory. An alternative explanation is that the substitutions at residues 308 and 311 do not affect the Cys-307 residue but the putative structurally important Lys-309 residue.

Variation in the virulence characteristics of escape variants *in vivo* can be associated with the substitution responsible for the antigenic properties. However, the presence of additional substitutions in other areas of the genome which may be important as genetic determinants of virulence cannot be ruled out. Nevertheless, the previous study of experimentally generated escape variants clearly illustrated the importance of residues 308 to 311 in the attenuation of the virus (Jiang *et al.,* 1993). LI/A was the most attenuated virus, and similar results were obtained in a previous investigation of this virus (Gao *et al.,* 1994). Presumably the amino acid substitution alters the conformation of the IgC-like module which is the putative site of receptor binding and in addition alters the structure of the epitope. INV6 virus exhibits pathogenicity traits similar to the wild-type virus SB/526, although like LI/A virus it encodes Glu at residue 308. Therefore the additional substitution of Lys-311 to Arg

may in some manner compensate for the substitution at residue 308 and limit the detrimental effect of this substitution on domain III in a manner similar to that described above for the WTBE virus infectious clone.

This situation may also account for the virulence characteristics of the viruses INV1 and DEV4. Both viruses are less attenuated than LI/A virus although they also encode Glu at residue 308, and INV1 virus is more pathogenic than the wild-type virus SB/526. The complete E gene sequences of these two viruses have been determined and show close similarity with LI/A virus. The deduced amino acid sequence of the E protein of INV1 virus only differs from that of LI/A virus at one residue, resulting from the substitution of Met-356 to Thr. This is a unique substitution and presumably alleviates the effect of the amino acid change at residue 308. The E protein sequence of DEV4 virus differs from that of LI/A virus at two residues. The residue Thr-172 is found within domain I and is unlikely to affect the conformation of domain III. The second amino acid difference involves the substitution of Ala-346 to Thr which lies within domain III. This substitution is unique to viruses from the south-west of England recovered after 1980.

LI/A virus was isolated in 1980 and is the least pathogenic type-II escape variant investigated to date. Later type-II escape variants, and INV6 virus, are more pathogenic and contain an additional substitution within domain III. Whatever pressure led to the selection of these antigenic phenotypes, the substitution at residue 308 resulted in the virus becoming less pathogenic and may have resulted in reduced levels of replication. By alleviating the conformational effect brought about by the presence of Glu at residue 308, the additional amino acid change may increase the fitness of the virus, possibly by increasing its replication ability. Therefore the amino acid change would become fixed in the population. At present insufficient information is available to investigate this theory.

The substitutions causing the escape variant phenotypes have been shown to be examples of Darwinian positive selection where nonsynonymous substitutions have occurred in the absence of observable synonymous substitutions (Zanotto *et al.*, 1995). However, the selection pressures involved are not known. Escape variants of hepatitis B virus and HIV have been selected for by immune pressure (Phillips *et al.*,

1991; Fujii *et al.,* 1992; Waters *et al.,* 1992; Ni *et al.,* 1995a). Hepatitis B escape variants which encode single amino acid substitutions at residues 144 or 145 of the surface antigen have been recovered from vaccinated children born to hepatitis B virus infected mothers. The amino acid substitutions Asn-144 to Thr/Ile and Gly-145 to Arg result in a substantial change in charge, size and hydrophobicity thereby altering the secondary and tertiary structure of the protein and several epitopes (Waters *et al.,* 1992; Ni *et al.,* 1995a). Substitutions within the Gag protein of HIV result in protein variability leading to the failure of cytotoxic-T cells to recognize virus infected cells (Phillips *et al.,* 1991).

LI virus escape variants may have been selected for because of the immune response or because they resulted in an improvement in the biological function of the virus and only incidentally affected epitopes (Both *et al.,* 1983; Domingo *et al.,* 1993). It appears that type-I and type-II escape variants are stable within the population, although revertants of escape variants of Sindbis (SIN) virus and poliovirus have been reported (Stec *et al.,* 1986; Ketterlinus *et al.,* 1993). Experimentally selected LI virus escape variants did not revert back to the parental virus phenotype when cultured in the absence of MAb 4.2 (Jiang *et al.,* 1993). The occurrence of naturally occurring escape variants supports this finding. All viruses from the south-west of England, collected over a fifteen year period, had the same antigenic characteristic.

The frequency of the occurrence of the various antigenic forms differs considerably in different areas of the British Isles and the occurrence of the two types of escape variants only overlaps in Scotland. However, the two viruses in question may have been recovered from distant regions due to the Inverness VI centre receiving samples from over a large area of the north of Scotland and the Western Isles. All viruses from Ireland exhibit wild-type characteristics while the only virus from Wales investigated to date is a type-I escape variant. Eleven viruses have been collected from the north of England and 27% of these are type-I escape variants. However, sixty percent of viruses collected after 1985 from the north of England are escape variants. All viruses from the south-west of England are type-II escape variants, while only one in 30 (3%) viruses from Scotland exhibited the type-II

escape variant phenotype. In addition 3% of viruses from Scotland were type-I escape variants.

The antigenic analysis of over fifty LI viruses has shown that the incidence of escape variants is much lower in Scotland than in England and Wales. The higher frequency of type-I escape variants from the north of England collected after 1985 implies that temporal and spatial elements are involved in the selection of the antigenic phenotypes. This may have resulted from a change in farming or vaccination practices, but this is not known at this time. It is also unclear why type-I escape variants are selected in some areas while type-II escape variants predominate in others.

Table 3.1. Grouping of LI viruses determined by IIF analysis using E and NS1 protein-specific MAbs raised against LI/31 virus (section 2.5). ■ indicates that fluorescence was observed and □ indicates that no fluorescence was seen. The MAbs F7/3 and 86.25 were used as a positive and negative control respectively. WT denotes wild-type virus, type-I and type-II denote type-I and type-II escape variants respectively.

Virus	MAb: E Protein-Specific 3.1	3.3	4.2	7.1	NS1 Protein-Specific 1.1	1.2	3.2	4.1	7.3	7.4	7.5	7.6	8.1	8.2	Controls F7/3	86.25	Group
LI/369	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
BRIS1	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-II
DEV1	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-II
DEV2	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-II
DEV3	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-II
DEV4	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-II
HUR1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV1	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-II
INV2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV4	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV6	■	■	□	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-I
INV7	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV8	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV9	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV10	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV11	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV12	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV13	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV14	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
INV15	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
IRE 1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
IRE2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
IRE3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
IRE4	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
LOCH1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
LOCH2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
LOCH3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
LOCH4	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
LOCH5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
LOCH6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
OBAN1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
PEN1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
PEN2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
PEN3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
PEN4	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
PEN5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
PEN6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
PRES1	■	■	□	□	■	■	■	■	■	■	■	■	■	■	■	□	Type-I
THK1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
THO1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
THO2	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT
WEST1	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	WT

Virus	Antigenic Phenotype	MAb 4.2	MAb 7.1
INV14	wild-type	-*	1.19
SB/526	wild-type	4.77	1.58
INV6	type-I	1.76	0.72
PRES1	type-I	2.56	0.66
DEV1	type-II	3.79	0.77
DEV2	type-II	3.79	0.74
DEV3	type-II	3.73	0.74
DEV4	type-II	3.92	0.75
INV1	type-II	3.63	0.69
LI/A	type-II	3.81	0.65

Table 3.2. Plaque neutralizing titres of MAbs 4.2 & 7.1 against 10 LI viruses. Results are expressed as the negative logarithm (log_{10}) of the end-point dilution of MAb (ascitic fluid) which reduced the plaque count by 75%. * indicates analysis was not carried out. The antigenic phenotypes were assigned according to the IIF analysis (Table 3.1).

a)

b)

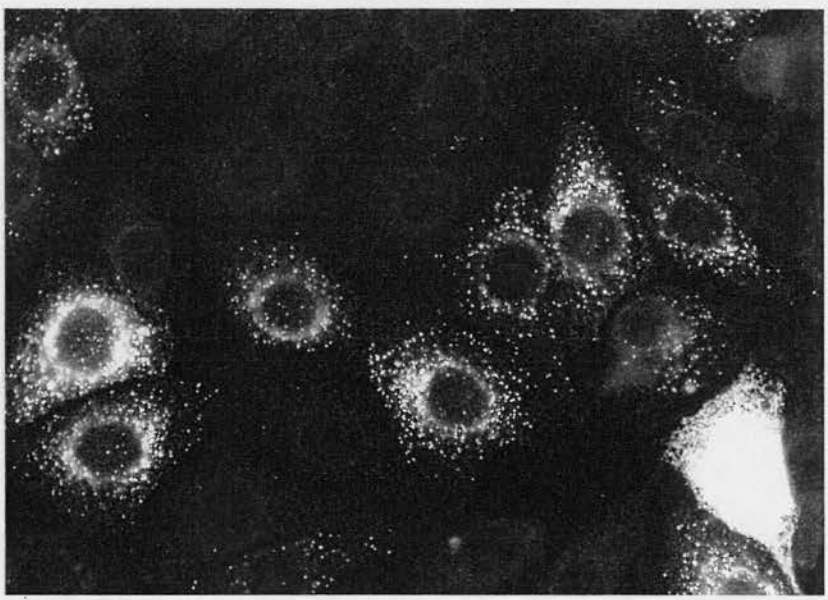


Fig. 3.1. Immunofluorescent staining of PS cells 48 hours post infection with the LI virus INV4 using; a) the E protein-specific MAb 4.2 and b) the NS1 protein-specific MAb 4.1 (x1320 magnification).

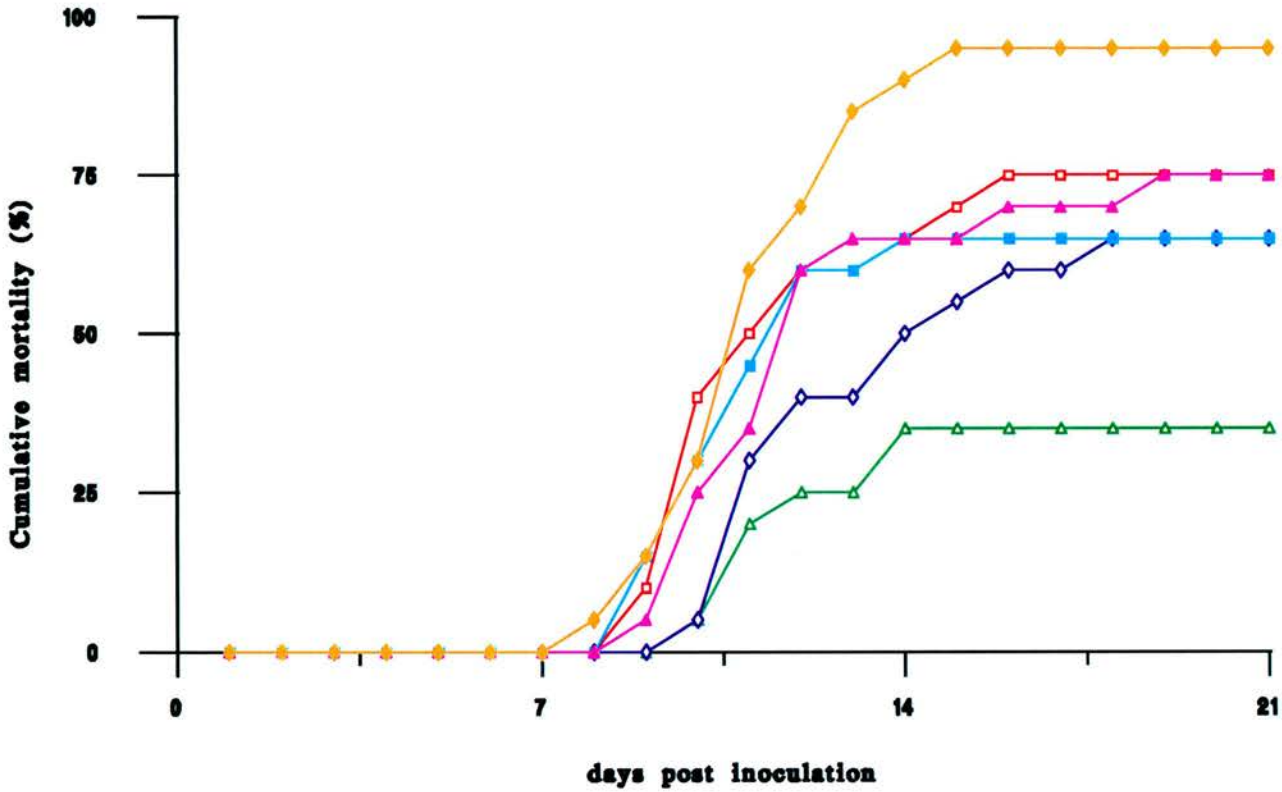


Fig. 3.2. Cumulative mortality of three week old C57/BL mice inoculated with type-I escape variants, INV6 (—■—) & PRES1 (—▲—) and type-II escape variants DEV4 (—◇—), INV1 (—◆—) and LI/A (—△—), compared with the wild-type virus SB/526 (—□—).

	293						*		370	Antigenic Type
Li-369	GLEKLKMKGL	TYTMCDKSKF	AWKRTPTDSG	HDTVVMEVTF	SGSKPCRIPV	RAVAHGSPDV	NVAMLITPNP	TIENDGGG		WT
LI-I	....	........Q.	....	....	....	....	D.........	....		Type-I
LI-261	....	........R.	....	....	....	....	....	....		Type-I
Li-917	....	........R.	....	....	....	....	....	....		Type-I
Inv6	....	.....E..R.	....	....	....	....	....	....		Type-I
Pres1	....	........R.	....	....	....	....	....	....		Type-I
LI-A	....	.....E....	....	....	....	....	....	....		Type-II
Bris1	....	.....E....	....	....	....	...T......	....	....		Type-II
Dev1	....	.....E....	....	....	....	...T......	....	....		Type-II
Dev2	....	.....E....	....	....	....	...T......	....	....		Type-II
Dev3	....	.....E....	....	....	....	...T......	....	....		Type-II
Dev4	....	.....E....	....	....	....	...T......	....	....		Type-II
Inv1	....	.....E....	....	....	....	....	...T......	....		Type-II

Fig. 3.3. Multiple alignment of the deduced amino acid sequences of type-I and type-II escape variants of LI virus. The alignment includes residues 293-370. The antigenic phenotype classification was assigned according to the IIF analysis. WT denotes wild-type virus, type-I and type-II denote type-I and type-II escape variants respectively. Residues involved in the antigenic characteristics of the viruses are overlined. * denotes amino acid substitution proposed to alleviate the detrimental effect of the substitution at residue 308. The single amino acid code has been used.

CHAPTER FOUR

Tracing the Origins and Evolution of Louping-ill Virus by Molecular Phylogenetic Analysis

CONTENTS

4.1. Introduction

Monoclonal antibody and other types of immunological analyses can only identify phenotypic changes in organisms which may arise from one or more unrelated genetic changes. Genotypic changes occur as nucleotide substitutions, the majority of which arise in the silent, third position of the codon. Therefore most variation among isolates of a single flavivirus species may be phenotypically invisible and only become apparent by comparing nucleotide sequences. This is illustrated by the results in the previous chapter. LI viruses were separated into three groups by MAb analysis, and the groups showed little correlation with the geographic distribution of the viruses. In particular, type-I escape variants were isolated in England, Scotland and Wales. However, sequence analysis showed a more complex situation in which amino acid substitutions causing the antigenic characteristics were associated with geographic distribution. This highlights the advantage of sequence analysis compared with antigenic analysis when investigating closely related viruses.

A number of studies have been carried out to investigate the variation among isolates of several flavivirus species. RNA oligonucleotide fingerprinting of 57 SLE viruses collected from across North America identified three topotypes, i.e. groups of viruses associated with particular geographic areas (Trent *et al.*, 1981). MAb analysis of European and Scandinavian TBE viruses implied that little antigenic variation occurred (Stephenson *et al.,* 1984; Guirakhoo *et al.,* 1987). However, only a small number of viruses was investigated and MAb analysis may not detect differences between closely related viruses, for the reasons discussed above. Several studies have investigated variation among DEN viruses, especially DEN type-2 virus, by different methods including; RNA oligonucleotide fingerprinting (Trent *et al.,* 1989), RNA-DNA hybridization (Kerschner *et al.,* 1986) and sequence analysis (Rico-Hesse, 1990; Blok *et al.,* 1989, 1991; Deubel *et al.,* 1993; Lewis *et al.,* 1993; Lanciotti *et al.,* 1994). It is clear from these studies that variation occurs at a relatively high rate compared with tick-borne flaviviruses.

Several molecular epizootiological studies have been carried out on the flaviviruses and alphaviruses which are enzootic in Australia (MacKenzie *et al.,*

1996b). The flaviviruses KUN and MVE and the alphavirus SIN virus exhibit relatively little genetic variation (Coelen & MacKenzie, 1988; Flynn *et al.,* 1989; Sammels, 1995). However, topotypes of the alphavirus Ross River (RR) have been detected by RNA oligonucleotide fingerprinting and sequence analysis (Lindsay *et al.,* 1993; Sammels *et al.,* 1995). The differences in diversity relate to the different epizootiological patterns of these viruses. The principal vertebrate hosts of MVE, KUN and SIN virus are numerous species of migratory waterbirds which disperse the viruses widely resulting in homogeneous virus populations. In contrast, the main vertebrate hosts of RR virus are non-migratory terrestrial animals and therefore there is little dispersal of the virus and microevolution of geographically isolated viruses leads to the emergence of topotypes. A few exceptions were identified with viruses of one topotype being found in an area primarily associated with another topotype. This most probably results from the rapid movement of infected humans or livestock over large distances (Sammels, 1995).

The principal vertebrate hosts of LI virus are non-migratory mammals and birds, e.g. sheep and grouse. Moreover, the vector *I. ricinus* does not move horizontally over large distances except when feeding on a moving host. Therefore a situation similar to that of RR virus is expected to arise, with LI viruses exhibiting microevolution within geographically isolated virus populations.

The tick-borne flaviviruses are genetically closely related and show a clinal distribution, the more ancient virus lineages being found in the east, i.e. Asia, and the most recent, represented by LI virus, being found in the west (Zanotto *et al.,* 1995). Details of the evolution of LI virus at the end of this cline have not hitherto been reported. MAb analysis has identified a number of viruses indistinguishable from LI virus, which cause sheep encephalomyelitis in Spain, Norway, Turkey, Greece and Bulgaria (Pavlov, 1968; Hartley, 1969; Papadopoulos *et al.,* 1971; Gonzalez *et al.,* 1987). However, sequence analysis has shown that only viruses isolated in the British Isles and Norway were genetically very closely related (Gao *et al.,* 1993a, b; Whitby *et al.,* 1993b; Marin *et al.,* 1995a). The Spanish, Turkish, Greek and Bulgarian viruses are significantly different. Furthermore, Negishi (NEG) virus, reportedly isolated in Japan in 1948 during a JE virus epidemic, was shown by

antigenic analysis to be closely related to LI virus and this was confirmed when the E gene sequence of NEG virus was determined (Venugopal *et al.,* 1992). NEG virus and LI/369, a LI virus from Scotland, share 97.3% nucleotide and 98.2% amino acid sequence similarity, demonstrating that NEG is a strain of LI virus (Venugopal *et al.,* 1992). The mechanism by which NEG virus (or more correctly LI virus) could have reached Japan is unknown although the involvement of migratory birds has been proposed. Since only a single strain of NEG virus is available for analysis and there have been no subsequent isolations of the virus, it is not unreasonable to suggest that contamination of the sample with LI virus could have occurred during the initial characterization of the virus (Zanotto *et al.,* 1995). In view of these discrepancies NEG virus is not included in the phylogenetic analysis of this study.

To date sequence data are available for the complete E genes of eleven LI viruses (Shiu *et al.,* 1991; Venugopal *et al.,* 1992; Gao *et al.,* 1993b; Gao, 1995). Phylogenetic analysis of these sequence data illustrates that variation among LI viruses occurs, which could not be detected by MAb analysis. The Irish virus, MA54, was shown to be distinct from other LI viruses, sharing 95.4-96.4% amino acid sequence similarity compared with 97.6-98.8% amino acid sequence similarity between other LI viruses. These results imply that MA54 virus may represent a distinct flavivirus (Gao, 1995), which is further considered in this study.

In the study by Gao (1995) the number of LI viruses investigated was limited, in particular only one virus from Ireland and Wales was included in the study. The viruses investigated may not have been representative of the virus in that geographic area. Therefore to obtain more meaningful data concerning the variation and evolution of LI virus in the British Isles, a larger sample of viruses needs to be investigated. In the study reported here, nucleotide sequence analysis was carried out with a large number of viruses from areas of the British Isles where LI virus is enzootic. A number of viruses was obtained from each area to look for evidence of microevolution within isolated virus microcommunities.

At the time the work was carried out, sequencing the entire genome or even a complete gene was still time consuming and impractical for analyzing a large number of samples. Therefore, in this study a representative gene fragment (RGF) was

chosen for analysis which fulfilled several criteria. Since analysis of the genetic markers is one of the aims of this study the RGF was designed to include the species-specific tripeptide and tick-borne specific hexapeptide markers (Shiu *et al.*, 1991, 1992a). The region chosen had been shown previously to contain a hypervariable domain (Gritsun *et al.*, 1995). Available sequence data were used to see whether or not phylogenetic analysis of the RGF enabled the same conclusions to be drawn as analysis of the complete gene or genome. A region 322 nucleotides in length was chosen as the RGF (termed region 3 in Table 2.3). This region contained nucleotides 525-846 encoding amino acids 176-282. The RGF comprised 21.7% of the E gene and 3.0% of the complete genome.

All 43 viruses included in this study had low passage histories which minimized laboratory induced nucleotide substitutions that might affect the phylogenetic analysis. The PCR product obtained from each virus was sequenced by direct solid-phase sequencing without cloning. Therefore the sequences determined for each virus comprised the consensus sequence of the quasispecies virus population present in the sample (Lewis *et al.*, 1992). This method was more rapid than others involving cloning, which would require the additional sequencing of more than one clone to ensure a representative sequence was obtained.

The RGF sequence of 42 out of the 43 LI viruses included in this study was determined. In addition, the complete E genes of 9 LI viruses were determined to provide additional data to investigate LI virus variation and provide more insight into the evolution of LI virus.

4.2. Results

4.2.1. Molecular Variation in the Representative Gene Fragment

Forty two of the 43 available LI viruses were sequenced across the RGF of the E gene. Multiple alignments of the nucleotide and deduced amino acid sequences were constructed and are recorded in Appendices 4.1 and 4.2 respectively. The sequences

of previously investigated LI viruses were included to extend the analyses. There was little evidence of sequence heterogeneity among the quasispecies present in the virus samples. The only exception was the isolate IRE4 which contained two distinct sequences (Fig. 4.1). Because of the heterogeneous population of virus in IRE4 the sequence of the RGF was not determined, and therefore sequence data from 42 out of the possible 43 viruses were incorporated in this study.

The multiple alignment of the nucleotide sequences of 53 LI viruses identified a number of substitutions. Within the RGF there was a total of 86 sites of substitution. Of these 76.7% occurred at the third position of the codon, while 14.0% and 9.3% occurred at the first and second positions respectively. The RGF contained conserved and variable regions. For example, nucleotides 675-715 were more variable than nucleotides 745-775. There were no insertions or deletions in the sequences. Several nucleotide substitutions were unique to viruses from particular areas. For example, cytosine at position 598 of the E gene was only detected in viruses from the south-west of England, while adenine at position 642 only occurred in viruses from Penrith.

Appendix 4.2 clearly illustrates that there was little amino acid sequence variation among these LI viruses. Substitutions at residues; Tyr-179 to Phe, Leu-184 to Met, Gln-214 to Arg, Asn-221 to Asp, Ala-238 to Val, Lys-251 to Arg and Tyr-281 to His were unique to INV15, LI/917, LI/G, PRES1, LI/31, LI/I and OBAN1 virus respectively. Other amino acid substitutions were observed in more than one virus. The substitution of Ile-198 to Val occurred in INV10, INV4, IRE1, IRE2 and MA54 virus. All viruses encoded the residue Asp at position 230, except LI/31 and SB/526 virus which encoded a Glu residue at this site, and LI/I virus which had a Val residue. The substitution of Lys-266 to Arg was observed in LI/369, IRE3, INV7, INV8, INV9, INV14 and LOCH4 virus.

Two genetic markers were present in the RGF. The hexapeptide serocomplex marker was present and identical in all 53 LI viruses sequenced to date. However, three forms of the tripeptide, species-specific marker were identified. Three of the four Irish viruses investigated encoded the marker GPR at residues 232-234. The remaining Irish virus, IRE3, encoded the genetic marker NPH, as did the majority of

viruses from England, Scotland and Wales. However, 10 viruses encoded the marker NPY, with the substitution of His with Tyr at residue 234. These viruses; PEN1, PEN2, PEN4, PEN5, PEN6, THK1, WEST1, INV2, INV3 and INV6 were collected in two geographic areas, from the north of England and the Inverness region of Scotland. Viruses encoding the NPY genetic marker were collected from sheep and grouse brain tissue between 1983 and 1993.

Phylogenetic analysis of the RGF produced the maximum likelihood tree illustrated in Fig. 4.2. The analysis included closely related TBE serocomplex flaviviruses and was rooted using the sequence of the Sofjin strain of FETBE virus. The phylogenetic analysis clearly distinguishes between the different flavivirus species. Three of the four Irish viruses, those with the genetic marker GPR, are also distinct from other LI viruses, except the Welsh LI/I virus. The remaining LI viruses from Scotland, England, Norway and IRE3 from Ireland are closely related, indicated by the short horizontal branch lengths and the low bootstrap values (those under 75% are not included in the figure). However, two Scottish viruses, LI/31 and SB/526, represent a separate lineage. Within the large group of viruses there is evidence of an association between genetic variation and geographic distribution with viruses from particular locations tending to cluster together, e.g. Penrith, Lochindorb and South-West England.

The only exception to the relationship between genetic variation and geographic distribution is IRE3 virus from Ireland. The RGF sequence of IRE3 virus was distinct from other Irish viruses, sharing 88.5-90.1% nucleotide sequence similarity compared with 98.4-99.1% nucleotide sequence similarity exhibited between IRE1, IRE2 and MA54 virus. IRE3 virus shared closer sequence identity with LI/369, LI/G and OBAN1 virus which were collected in the west of Scotland. The RGF of a fourth isolate from Ireland, IRE4, was sequenced and the sample was found to contain two virus populations distinguished by the presence of sequence encoding for both the NPH and GPR genetic markers (Fig. 4.1).

4.2.2. Molecular Variation in the Complete E Gene

As shown above, phylogenetic analysis of the RGF has extended our understanding of LI virus variation in the British Isles. However, the variation detected between many of the viruses, especially those in Scotland, is so low that the information obtainable from the sequence data is limited. Therefore the complete E gene sequences of nine LI viruses with potentially interesting phenotypic characteristics were determined. These viruses were; DEV4, INV1, INV6, INV14, IRE3, PEN3, PEN6, THO1 and THO2.

Multiple alignments of the determined nucleotide and deduced amino acid sequences are shown in Appendices 4.3 and 4.4 respectively. The sequence data available for previously investigated LI viruses were included to extend the analysis. Table 4.1. summarizes the percentage sequence similarities calculated for these viruses.

Among the 20 LI viruses there was complete conservation of the 12 cysteine residues and the putative fusion peptide situated at residues 98-111. The potential glycosylation sites within the E protein, at Asn-154, Asn-361 and Asn-473, were also conserved in all the LI viruses investigated. In addition, the potential T-cell determinant region of the E protein, residues 398-413 (Mandl *et al.*, 1989a), was highly conserved among LI viruses. A single substitution of Thr-405 to Lys occurred in SB/526 virus.

Several viruses shared a considerable degree of sequence similarity. PEN3 and PEN6 virus, which were isolated from the same farm, differed at only four nucleotides of which two are the result of nonsynonymous substitutions. One of the amino acid substitutions resulted in the altered species-specific genetic marker. In addition, the Devon viruses, LI/A and DEV4, shared greatest nucleotide sequence similarity with; THO1, THO2, INV6, INV1 and LI/K virus, which were all collected in the east of Scotland.

The results summarized above are more clearly illustrated on the maximum likelihood phylogenetic tree generated from the complete E gene nucleotide sequence data (Fig. 4.3). Closely related flaviviruses have been included in the phylogenetic

analysis. The tree is in general agreement with that produced using the RGF sequence data (Fig. 4.2) and the different flavivirus species are clearly distinct from one another. A major difference between the two trees relates to the Welsh virus, LI/I. Phylogenetic analysis of the complete E gene sequence implies that LI/I virus represents a distinct viral lineage and high bootstrap values support this conclusion.

The Irish virus IRE3 shared only 92.3% and 96.4% nucleotide and amino acid sequence similarity respectively with another Irish virus MA54. IRE3 virus clustered on the phylogenetic tree with viruses from the west of Scotland supporting the suggestion that two distinct viruses are present in Ireland.

The divergence of LI viruses depicted on the phylogenetic tree can be interpreted to describe the spatial dynamics of LI virus evolution and dispersal (Fig. 4.4). The most divergent LI virus lineage is represented by MA54 virus from Ireland, followed by LI/I virus from Wales, which diverged before viruses from England and Scotland. These results imply that LI virus originally emerged in Ireland and was introduced into Great Britain, via Wales, at a later date. The next most divergent virus lineage is represented by LI/31 and SB/526 virus, both from Scotland. All remaining Scottish, English, Norwegian and Irish viruses descend from a common ancestor with LI/31 and SB/526 virus. This relationship implies that the virus was transported from Wales to Scotland from where it was dispersed throughout Scotland, Northern England and was introduced into Norway. More recently the virus was reintroduced into Ireland, resulting in the presence of two distinct viruses. In addition, the virus was introduced into Devon, probably from the east of Scotland.

4.2.3 Estimation of the Rate of Synonymous Substitution & Lineage Divergence Times

Pairwise comparisons of the E gene sequences of LI viruses from particular geographic areas suggest that there is a relatively low rate of nucleotide substitution in these viruses. DEV4 and LI/A virus were collected fifteen years apart but differ at only three synonymous and two nonsynonymous sites. Moreover, THO1 and THO2

viruses differ at only one synonymous site across the entire E gene, even though THO2 virus was collected six years after THO1 virus.

The date of isolation is known for all viruses included in this study. Therefore, by using the method developed by Li *et al.* (1988) and described in section 2.13 the rate of nucleotide substitutions can be estimated. This method of calculation was used because it recognizes that the time of viral isolation is not the same as the time of lineage divergence. Because of the close phylogenetic relationship and the relatively low number of nonsynonymous substitutions occurring between these viruses, the rate of synonymous substitutions was calculated from the available complete E gene sequence data. Nevertheless, the genetic distances between the viruses investigated were so small that a wide range of rate values were calculated, including negative values. For example, Fig. 4.2 implies that LI/A virus has evolved further than DEV4 virus despite being isolated prior to DEV4 virus.

The rate calculation method used in this study depends upon the topology of the phylogenetic tree, which is uncertain in places. Therefore in an attempt to estimate a more accurate synonymous substitution rate, a phylogenetic tree was constructed which highlighted the relationships between viruses which were highly supported by bootstrap values exceeding 75% (Fig. 4.5). In other words, rates were only calculated between sequences for which the phylogenetic relationships could be estimated with some confidence. In addition, each rate value was calculated independently, i.e. each LI virus was only used in one calculation, to reduce the cumulative effect of stochastic errors and prevent the analysis being biased towards particular lineages. Furthermore, SSE virus was chosen as the outgroup for each calculation as the relatively large genetic distances between SSE and LI viruses further reduced the influence of stochastic errors which are more likely to occur in very closely related sequences. These precautions resulted in the calculation of nine rate values, listed in Table 4.2. A large range of values were calculated including some with negative values which clearly are biologically impossible and therefore in reality reflect the extent of rate variation. However, the average synonymous substitution rate was estimated to be 4.76×10^{-4} substitutions/site/year with a standard error of 8.03×10^{-4} substitutions/site/year.

If it is assumed that the nucleotide substitution rate prior to isolation was constant and that after isolation, no further nucleotide substitutions occurred, then the approximate time of viral lineage divergence can be estimated to provide an indication of the temporal dynamics of LI virus evolution. Table 4.3 contains the estimates of lineage divergence calculated using the mean average synonymous substitution rate. The extremely large standard error calculated for the synonymous substitution rate illustrates the inherent difficulties involved in making such estimates from a small sample of very closely related viruses. Therefore the lineage divergence times calculated in this study should be used only as a guide to indicate the time frame of the divergence events and more data are needed in the future to confirm the results of this analysis.

The estimated lineage divergence times calculated using the average synonymous substitution rate combined with the phylogenetic analysis demonstrate that LI virus emerged relatively recently. SSE virus represents the mainland European virus most closely related to LI virus and the two lineages are estimated to have diverged approximately 500 years ago. Whether or not this divergence resulted from the introduction of the ancestral virus into Ireland is not known, but the estimate of 500 years provides a rough guide for the time of its introduction into the British Isles. The Irish and Welsh virus lineages, represented by MA54 and LI/I virus respectively, are estimated to have diverged about 300-400 years ago, which is similar to that estimated for the divergence of lineages represented by LI/I and LI/31 virus. Most of the LI virus dispersal is estimated to have occurred 100-200 years ago with the emergence of English, Scottish and Norwegian viruses. More recent events include the divergence of lineages represented by LI/369 virus from the west of Scotland and IRE3 virus from Ireland, which are estimated to have diverged 100 years ago. The emergence of the Devon viruses was shown on the phylogenetic trees to be the most recent divergence event which, using the substitution rate estimated here, probably occurred during this century.

4.3. Discussion

4.3.1. Genetic Variation Among LI Viruses

MAb analysis of the LI viruses included in this study identified three groups of viruses which could not be further subdivided. The classification based on MAb analysis showed little relation to the geographical distribution of viruses, with LI/I, LI/261 and INV6 virus from Wales, Northern England and Scotland respectively being grouped together. Moreover, the nucleotide substitutions that caused each antigenic characteristic were different for each virus. Phylogenetic analysis of sequence data presents a very different perspective and enables a more detailed analysis of the relationship between geographic distribution and genetic variation of virus populations.

To investigate a large number of viruses a relatively short region of the E gene, 322 nucleotides in length, was studied. Several previous studies investigating the phylogenetic intrarelationships of flaviviruses have concentrated on sequencing a fragment of the genome (Rico-Hesse, 1990; Chungue *et al.,* 1993; Deubel *et al.,* 1993; Lepiniec *et al.,* 1994). These studies determined various fragments of the E gene from 195-348 nucleotides in length. However, Lewis *et al.* (1993) argued that because of the high sequence similarities between isolates of a single flavivirus species any phylogenetic analysis must involve a large number of nucleotides to obtain reliable conclusions. To some extent this problem can be overcome with the choice of the fragment used for analysis. Comparison of the maximum likelihood trees produced by phylogenetic analysis of the RGF and the complete E genes of LI viruses (Figs. 4.2 and 4.3 respectively) illustrates that analysis of the shorter region provided similar information to analysis of the longer sequence and the data are therefore phylogenetically robust. In addition, there is a strong positive correlation between the percentage nucleotide similarities generated between LI viruses using the RGF and complete E gene sequence data ($r = 0.89$, Fig. 4.6). Analysis of the RGF clearly distinguishes between different flavivirus species. Therefore the determination of this small region of sequence, which can be achieved in a matter of

days, provides a useful tool for the study of flavivirus dispersal and also for the diagnosis of individual species.

The main difference between the two phylogenetic trees concerns the Welsh virus LI/I. Phylogenetic analysis of the RGF implies that there is a closer relationship between the Welsh and Irish viruses than was suggested by the complete E gene analysis, although this is not supported by bootstrap analysis (Fig. 4.2). The tree topology is largely due to the fact that the Welsh virus has a relatively high proportion of substitutions in the RGF. Pairwise comparison of the entire E gene sequence of LI/31 and LI/I virus identifies 32% of the nucleotide differences as occurring within the RGF, which comprises only 22% of the E gene. In contrast, pairwise analysis of LI/31 and MA54 virus identifies 24% of substitutions as having occurred within the RGF. This illustrates one limitation of analyzing a relatively short region of sequence.

The LI viruses investigated are so closely related that relatively few amino acid substitutions were detected, even within the entire E gene. Therefore phylogenetic analysis was carried out on the nucleotide sequence data which provided more information concerning the variation among LI viruses. The disadvantage of nonsynonymous sequence analysis is highlighted by the relationship between LI/A, INV1 and DEV4 virus. LI/A and DEV4 virus share 99.7% and 99.6% nucleotide and amino acid sequence similarity respectively. LI/A and INV1 virus share 98.9% nucleotide sequence identity but 99.8% amino acid sequence similarity. Therefore the nonsynonymous sequence data imply that LI/A virus is more closely related to INV1 virus than DEV4 virus, which is clearly not supported by the nucleotide sequence data. The relatively low level of amino acid substitutions observed between LI viruses means that single substitutions can have a profound effect on the interpretation of data.

Fig. 4.3 suggests that three distinct geographic populations of LI virus are present in the British Isles, represented by MA54 virus from Ireland, LI/I virus from Wales and finally the large group of viruses from England, Scotland, Norway and IRE3 virus from Ireland. These groupings were not correlated with those identified by MAb analysis. The Devon viruses, LI/A and DEV4, appear on the maximum

likelihood phylogenetic tree (Fig. 4.3) to be most closely related to viruses from the east of Scotland. This is supported by MAb analysis whereby type-II escape variants, with identical substitutions, were collected in the south-west of England and the Inverness region of Scotland.

The genetic variation among MA54, IRE1, IRE2 and IRE3 virus implies that two distinct virus populations exist in Ireland. The presence of both populations in the IRE4 isolate, which originated from a pool of *I. ricinus* ticks removed from a suspected case of bovine LI, implies that the two viruses co-inhabit the same tick population and niche. The presence of two virus populations in IRE4 and the identification of IRE3 as a Scottish-like virus are unlikely to be due to laboratory contamination since two separate samples, collected four years apart, are involved. In addition, IRE3 virus has a unique E gene sequence, sharing 98.3% nucleotide sequence similarity with the most closely related virus, LI/369, which also argues against laboratory contamination.

Within the large group of LI viruses there is some evidence of geographical clustering of viruses. All viruses from the south-west of England and Penrith had unique nucleotide substitutions within their E genes at positions 598 and 642 respectively. LI viruses recovered from the Lochindorb estate share 98.8-100.0% nucleotide sequence similarity. A previous study of eleven LI viruses from this estate found a similar degree of genetic homogeneity across a 240 nucleotide region of the E gene (Gaunt *et al.*, 1997). The sequences determined in this and the previous study overlap by 120 nucleotides and the 17 Lochindorb viruses share 99.2-100.0% nucleotide sequence similarity. Geographical clustering of viruses is not surprising for a tick-borne virus with non-migratory hosts. Introduction of ticks and virus into new areas is sporadic and thereafter the virus replicates in isolation leading to microevolution of viruses in each area. A similar phenomenon is observed with RR virus in Australia (Sammels, 1995). The movement of livestock and infected humans have been implicated in the occurrence of topotypes of RR virus in different geographic locations, similar to the presence of IRE3 virus in Ireland (Sammels *et al.*, 1995).

The genetic variation detected among LI viruses and the phylogenetic analysis carried out on these data enable the formulation of a theoretical dispersal pattern for LI virus evolution (Fig. 4.4). This involves the initial introduction of a European ancestral virus into Ireland. A descendant virus was later introduced from Ireland into Britain, via Wales. From this location the virus was dispersed to Scotland from where it radiated out and dispersed throughout Northern England and Scotland as well as into Norway. More recently the virus was reintroduced into Ireland from the west of Scotland and introduced into Devon from the east of Scotland.

4.3.2. Irish LI virus - A New Species of Flavivirus?

Phylogenetic analysis of the sequence data generated in this and previous studies implies that the Irish viruses; MA54, IRE1 and IRE2, represent a virus population distinct from LI viruses found in Scotland, England and IRE3 virus which was also isolated in Ireland. To a lesser extent the Welsh virus, LI/I, also appears to represent a distinct virus lineage. An attempt was therefore made to decide whether or not these viruses represent separate species of flavivirus.

The ICTV has defined a virus species as "a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecological niche" (Regenmortel *et al.*, 1991). A polythetic class consists of viruses which share a number of properties but do not share a single defining property which is found only in these viruses and no others. Viruses within a species comprise a monophyletic group with a shared genealogy. Furthermore, the viruses share biological properties such as; geographic distribution, host range, cell tropism, pathogenesis and vector tropism. Therefore to investigate whether Irish and Welsh LI viruses represent separate virus species a number of characteristics were studied including; genome sequence relatedness, natural host range, vector tropism, cell and tissue tropism and antigenic properties (Regenmortel *et al.*, 1997).

Members of the TBE virus serocomplex found in Western Europe evolved from a common ancestor relatively recently and therefore share high sequence similarities.

Consequently the demarcation between intraspecies and interspecies variation is difficult to define. Table 4.4 summarizes the pairwise nucleotide and deduced amino acid E gene sequence similarities for Asian and European members of the TBE virus serocomplex. The highest sequence similarity between members of the TBE virus serocomplex was observed between SSE and LI viruses which share 90.9% and 96.2% nucleotide and amino acid sequence similarity respectively. In contrast, intraspecies variation among isolates of WTBE, FETBE, TSE and LI viruses (excluding Irish and Welsh viruses) ranges from 94.6-99.9% and 97.8-100.0% nucleotide and amino acid sequence similarity respectively. The absence of any overlap in these two data sets can be seen in Figs. 4.7 and 4.8. Therefore the data included here identify a cut-off point between intraspecies and interspecies variation of between 91-93% nucleotide sequence similarity and 97% amino acid sequence similarity. The latter value is similar to the 2% amino acid sequence variation previously reported between isolates of the same flavivirus species (Heinz *et al.*, 1990). Nevertheless it cannot be over emphasized that these values are rudimentary as few complete E gene sequences have been determined for TBE serocomplex viruses other than LI virus.

Pairwise nucleotide sequence similarities for LI viruses, including Irish and Welsh viruses, lie within the cut-off margin between intraspecies and interspecies variation proposed above. By comparing the amino acid sequences, the Irish viruses lie outside the cut-off value of 97% implying that the viruses represent a separate virus species. However, the Welsh virus shares greater sequence similarity with other LI viruses than the demarcation value, suggesting that LI/I virus does not represent a separate virus species.

The epizootiology of the Irish viruses has not been studied extensively but appears to be similar to that of LI virus in Britain. The same vector is involved in the transmission of both virus populations. In addition, the host range does not vary, although the range of potential hosts may be wider for Irish viruses due to the occurrence of *I. ricinus* on better quality pasture in Ireland (Walton & Kennedy, 1966; Walton, 1967). During this study no evidence of antigenic variation between LI viruses from Ireland and Britain was identified. Moreover, there has been no

evidence of differences in cell and tissue tropism, although the Irish viruses were generally more pathogenic in laboratory mice and produce larger plaques than British LI viruses (chapter 5).

Taking all the above points into consideration it becomes clear that it is inappropriate to describe the Irish and Welsh viruses as separate virus species. Nonetheless, these viruses represent phylogenetically distinct virus populations and therefore could be described as topotypes of LI virus. However, the remaining large group of viruses is found over a wide geographical area including Britain, Ireland and Norway, and consequently the term topotype is also inappropriate. Therefore the three LI virus populations, represented by; the Welsh virus LI/I; the Irish viruses MA54, IRE1 and IRE2; and the remaining viruses from Ireland, Britain and Norway will be described as 'Welsh', 'Irish' and 'British' LI viruses respectively.

4.3.3. Genetic Markers

The hexapeptide and pentapepide genetic markers, which identify the TBE serocomplex flaviviruses (Shiu *et al.*, 1991; Gao *et al.*, 1993a), are present and identical in all LI viruses. However, variation was detected in the tripeptide marker at residues 232-234 of the E protein. The tripeptide GPR was identified in all Irish viruses except IRE3 virus. The remaining LI viruses had the marker NPH or NPY. British LI viruses with the NPY or NPH marker did not differ significantly and do not represent separate virus populations. In fact, PEN3 and PEN6 virus, which encode the genetic markers NPH and NPY respectively, were isolated from the same farm and differ at only 4 nucleotide sites within the entire E gene. Furthermore, the phylogenetic analysis of the Penrith viruses (Fig. 4.2) implies that an ancestral virus encoded the altered genetic marker NPY, which in the case of PEN3 virus reverted back to NPH.

The biological significance of the substitution of His to Tyr at residue 234 is not known. The change is not conserved except that both amino acids contain ringed structures. Therefore the substitution of one large molecule for another may limit

any physicochemically induced conformational change. The AQN tripeptide of TBE virus was shown to be involved in the binding of MAb A4 (Mandl *et al.*, 1989a) and therefore the presence of NPY and NPH may have biological significance or change the antigenic characteristic of the viruses in a manner undetected by the MAb panel used in this study. A substitution in the third residue of the tripeptide was also observed in the Vasilchenko (Vs) strain of TBE virus. Other TBE viruses encode the marker AQN while the Vs strain has the genetic code AQQ (Gritsun *et al.*, 1993a). It is interesting that the Vs strain is an attenuated strain of TBE virus and this will be discussed in more detail in the next chapter.

One of the aims of this thesis was to investigate the potential use of genetic markers in the classification of flaviviruses. The results imply that the use of the tripeptide species-specific marker is unsuitable for identification of different LI virus populations. The Welsh virus is phylogenetically distinct from British LI viruses but encodes the same genetic marker, NPH. However, if the genetic marker is extended to include residues 230-234, the resulting pentapeptide can be used to separate the LI viruses into four groups. The Irish viruses have the genetic marker DGGPR while the Welsh virus has the marker VGNPH. The British LI viruses can be divided into two groups; the genetic marker EGNPH is present in LI/31 and SB/526 virus, which represent a lineage quite distinct from other British LI viruses which encode the genetic markers DGNPH or DGNPY. Using this pentapeptide genetic marker, which was the species-specific marker initially proposed by Shiu *et al.* (1992a), the different LI virus populations can be distinguished. Therefore it appears that the tripeptide marker is suitable for the classification of different flavivirus species, but is unable to categorize more closely related viruses. However, the extension of the marker to a pentapeptide resolves this problem.

4.3.4. Temporal Dispersal Pattern of LI Virus Evolution

To investigate the temporal aspects of LI virus evolution the rate of nucleotide substitution was estimated. The close genetic relationship between LI viruses led to the estimation of the synonymous substitution rate using the method described by Li *et al.* (1988). Following the estimation of this value the times of lineage divergence were calculated. There are two assumptions for this model; firstly that the rate of substitution is constant over time and amongst all viruses examined and secondly that after isolation of the viruses no further substitutions have occurred during laboratory manipulation. The low passage history of the viruses investigated in this study limits the possibility of substitutions occurring after isolation. However, the assumption that a constant rate of substitution characterizes LI virus evolution cannot be verified. The nucleotide substitutions causing the escape variants are an example of Darwinian positive selection which accelerates the rate of substitution (Zanotto *et al.*, 1995). However, evolution of the E gene appears to have resulted from the accumulation of nucleotide substitutions with strong purifying selection of nonsynonymous substitutions so that selection is unlikely to cause variation in the synonymous substitution rate (Zanotto *et al.*, 1995). Although synonymous substitution rates are therefore more likely to be constant, it is clear from the data presented here that lineage-specific rate variation characterizes LI virus evolution. This rate variation most probably represents the small amount of evolutionary change that separates the viruses sampled, whereby stochastic effects have a disproportionately large effect on the rate estimations. This is also reflected in the large standard error estimated around the mean rate value ($4.76 \pm 8.03 \times 10^{-4}$ substitutions/site/year). Consequently, the average rate estimated is presented in this study as a hypothesis which needs to be tested with future analysis. Despite the rate variation observed, the estimated rate is compatible with a number of other pieces of evidence.

To investigate the suitability of the estimated substitution rate a comparison was made with the estimates published for other viruses. A large number of studies have been carried out using various techniques to investigate the nucleotide substitution rates of viruses, particularly those with RNA genomes. One method of estimating

rates uses regression analysis. The viruses being studied are compared to a marker sequence, either a hypothetical ancestor or an early isolated virus. The slope of the linear regression line, which illustrates the relationship between the genetic distance and the year of isolation, represents the average rate of sequence change (Buonagurio *et al.,* 1986). However, this method is not suitable for the sequence data generated in this study due to the majority of viruses being collected over a relatively short period of time, between 1979 and 1993. More simplistic methods have also been used to estimate substitution rates, including the pairwise comparison of closely related viruses isolated a known number of years apart (Weaver *et al.,* 1991). However, this results in single values being calculated which may not be representative of the viruses in question and the method assumes that the time of isolation is the same as the time of divergence. Therefore the method proposed by Li *et al.* (1988) was considered most appropriate for the dataset available in this study.

In a number of studies the substitution rate of HIV env and gag genes has been estimated. Several of these studies reported the synonymous substitution rate to be between 4.1×10^{-3} and 1.39×10^{-2} substitutions/site/year (Li *et al.,* 1988; Balfe *et al.,* 1990; Gojobori *et al.,* 1990), which is one or two orders of magnitude higher than that estimated in this study for LI virus. Similar substitution rates have been estimated for other RNA viruses. For example, the synonymous substitution rate of enterovirus 70 was reported to be 2.15×10^{-2} substitutions/site/year (Takeda *et al.,* 1994), while the synonymous substitution rate for hepatitis C virus was estimated to be between $1.1\text{-}1.7 \times 10^{-3}$ substitutions/site/year (Smith *et al.,* 1997). In addition, a nucleotide substitution rate of 2.3×10^{-3} substitutions/site/year was estimated for influenza A virus (Shu *et al.,* 1993). However, lower rate estimations were made for the alphaviruses. For the eastern equine encephalomyelitis virus complex the nucleotide substitution rate was estimated as $1.4\text{-}4.3 \times 10^{-4}$ substitutions/site/year (Weaver *et al.,* 1991, 1994), while for the western equine encephalomyelitis virus complex the rate was estimated to be between 9.0×10^{-5} and 2.8×10^{-4} substitutions/site/year (Cilnis *et al.,* 1996; Weaver *et al.,* 1997). Moreover, the nucleotide substitution rate for the Venezuelan equine encephalitis virus complex was estimated to be 5.0×10^{-4} substitutions/site/year (Weaver *et al.,* 1992). Since

these were nucleotide substitution rates the values cannot be directly compared with the synonymous substitution rate estimated for LI virus. However, in a typical sequence, synonymous substitutions represent approximately 25% of the nucleotide substitutions which are possible and therefore the synonymous substitution rate will be approximately four times higher than the nucleotide substitution rate (Simmonds, pers. com.). If this is assumed to be the case for the alphaviruses then the synonymous substitution rate would be between 3.6×10^{-4} and 2.3×10^{-3} substitutions/site/year. These estimates for the alphaviruses are situated between those for LI virus and non-arthropod borne viruses such as HIV.

One of the possible explanations for the variation in substitution rates of different viruses relates to their life-cycles and the involvement of invertebrate and/or vertebrate hosts. The two host system induces additional selective constraints on alphavirus and flavivirus evolution. In contrast, HIV, hepatitis C and influenza virus, which are not vector-borne, are likely to be subjected to less selective constraint leading to higher rates of evolution. Moreover, the fact that the estimated synonymous substitution rate for the mosquito-borne alphaviruses is higher than the average rate estimated for LI virus is not surprising in view of the greater evolutionary constraints imposed by the tick vector of LI virus and the lower turnover of virus associated with ticks.

Only two studies have investigated in depth the substitution rates in the flaviviruses (Zanotto *et al.,* 1996b; Lanciotti *et al.,* 1997). The former study estimated the rate of nonsynonymous substitution for the tick-borne flaviviruses as 4.1×10^{-5} substitutions/site/year (Zanotto *et al.*, 1996b). This value was extrapolated from the mosquito-borne flavivirus substitution rate of 7.5×10^{-5} substitutions/site/year, because tick-borne flaviviruses had previously been shown to be evolving 0.56 times as fast as mosquito-borne flaviviruses due to the constraints imposed by the tick vector (Zanotto *et al.*, 1995). This value and the nonsynonymous pairwise genetic distances generated by the MEGA software package (Kumar *et al.,* 1993) were used to calculate the time of lineage divergence for TBE and LI viruses. To summarize the findings, the study predicted that the TBE virus complex evolved along a cline during the past 2,000 years and LI virus emerged about 300 years ago

(Zanotto *et al.*, 1996b). Therefore the values calculated in this study using the mean synonymous substitution rate are similar to those reported by Zanotto *et al.* (1996b) although a different method of calculation was used. In the present study LI virus is estimated to have emerged approximately 500 years ago.

In addition to investigating the substitution rate for the tick-borne flaviviruses, Zanotto *et al.* (1996b) also estimated the non-synonymous substitution rate of a number of mosquito-borne flaviviruses. A value of 8.54×10^{-4} substitutions/site/year was estimated for DEN type-4 virus. However, only a limited dataset was used to generate this rate value. Using a different dataset and calculation method, a later study estimated the overall nucleotide substitution rate of DEN type-4 virus to be 8.3×10^{-4} substitutions/site/year (Lanciotti *et al.*, 1997). Unfortunately these values cannot be compared and additional studies investigating the substitution rate of the flaviviruses are not available.

The lineage divergence times calculated in this study differ slightly from those of Zanotto *et al.* (1996b) for two reasons. Firstly, Zanotto and co-authors (1996b) studied the rate of nonsynonymous substitutions which occur less frequently than synonymous substitutions. The point of substitution saturation, i.e. when substitutions become superimposed on top of one another, will be reached more rapidly with synonymous than nonsynonymous substitutions leading to an underestimation of the genetic distance between viruses. As a result the synonymous substitution rate is unsuitable for estimating the time of divergence of distant lineages. In contrast, due to the low rate of nonsynonymous substitutions, an order of magnitude lower than the synonymous substitution rate, this value is inappropriate for estimating the time of divergence of closely related lineages, for example between LI viruses. Secondly, in the previous study the nonsynonymous substitution rate was extrapolated from that of the mosquito-borne flaviviruses, due to the lack of available sequence data for tick-borne flaviviruses (Zanotto *et al.*, 1996b). In this study it was hoped that the availability of additional data would enable the calculation of a more accurate rate value for tick-borne flaviviruses. However, this was prevented by the extremely close genetic relationship between LI viruses. Nevertheless, the estimated rate provides further support for the recent emergence of the flaviviruses.

4.3.5. Possible Modes of Dispersal

The estimated times of lineage divergence calculated in this study imply that LI virus evolved from SSE virus approximately 500 years ago. The British Isles have been isolated from mainland Europe since the formation of the English Channel at the end of the last ice age, approximately 8,000 years ago. Therefore LI or the ancestral virus must have been introduced into the British Isles after the land masses (the British Isles and Europe) were separated. The phylogenetic analysis reported here suggests that the ancestral LI virus initially emerged in Ireland and was later introduced into Great Britain where it was dispersed to many isolated areas.

LI virus infection of vertebrate animals is transient, therefore the majority of LI virus dispersal presumably resulted from the movement of infected ticks. However, *I. ricinus* dispersal over large distances is dependent upon the movement of vertebrate hosts. Therefore there are three possible mechanisms by which LI virus may have been dispersed. Firstly, free-living terrestrial mammals, e.g. deer and mountain hares, may have transported feeding ticks to new areas. This may explain the local dissemination of LI virus from the point of origin but is unlikely to account for the movement of LI virus and infected ticks over large distances to isolated habitats, and does not explain the initial introduction of LI virus into the British Isles. Infestation of several bird species by *I. ricinus* has been reported (Milne, 1949) and bird migration is a more probable explanation for the transportation of infected ticks into new areas. The third and most likely mode of LI virus dispersal involves the movement of livestock. The predicted dispersal pattern and the relatively recent lineage divergence times calculated here implicate the involvement of man and agricultural practices. Sheep straying from one farm to another have been observed to introduce ticks onto new pasture (Milne, 1950). Moreover, the extensive movement of livestock across the British Isles would have provided ample and ideal opportunities for the dispersal of *I. ricinus* and LI virus.

The large error in the estimates of lineage divergence times prevents the identification of particular events which were responsible for the introduction and dispersal of LI virus. However, several events over the last 500 years, which

correlate approximately with the divergence dates calculated in this study, provide a suitable mechanism for the introduction or dispersal of LI virus to new regions. A few of these events have been summarized below.

Ireland has been an important producer of livestock, especially cattle, for hundreds of years. However, cattle are not native to Ireland and were introduced repeatedly through history from Great Britain and other countries in Europe (O'Donovan, 1940). Importation of livestock may therefore have resulted in the introduction of a flavivirus into Ireland. There has also been an extensive export trade in livestock from Ireland to England and Wales since the end of the 15th century (O'Donovan, 1940). During the 17th century the export of Irish livestock to countries other than England and Wales was banned and this included the importation of livestock into Scotland. This may explain why the virus was initially introduced into Wales.

The lineage divergence times calculated in this study suggest that after its introduction into Great Britain the virus was transported from Wales to the Borders of Scotland. At this time there was extensive movement of livestock across Great Britain, to and from market. Livestock, mainly cattle and sheep, were driven from Wales and Scotland to the markets of England by drovers from the 16th century until the advent of the railways (Bonser, 1970). Livestock markets in towns in Yorkshire were frequented by drovers from Wales and Scotland which may account for the movement of Welsh livestock into Scotland (Bonser, 1970). At the end of the 17th century there were heavy losses of sheep in the Borders of Scotland and new animals were brought into the area (Ryder, 1983). Unfortunately details of this event, such as the cause of the sheep mortality and the source of the replacement animals, are not known although it is believed that the replacement sheep came mainly from England. However, this event may explain the movement of LI virus from Wales to Scotland.

Prior to the middle of the 18th century most of the Scottish upland habitat was utilized as summer grazing for livestock, especially cattle, farmed by crofters. The only exception was in Southern Scotland where intensive sheep farming was introduced in the middle ages by Cistercian monks, who established the first Abbey at Melrose in 1136 (Ryder, 1983). In 1750 black-faced sheep, the main breed found in the Borders of Scotland, were introduced further north and then into the West

Highlands in 1762 (Scott & Scott, 1888). Sheep were found to be more profitable than cattle and intensive sheep farming spread throughout Scotland and the north of England. This brought about the Highland Clearances and may also have resulted in the widespread dispersal of LI virus from the Borders of Scotland which was estimated to have occurred 100-200 years ago. At the beginning of the 19th century Cheviot sheep were also introduced into the Highlands, from the Borders of England and Scotland (Wrighton, 1919), and this may also have played a role in the dispersal of LI virus. In the 19th century Cheviot sheep and other breeds were imported into Norway, from Britain, to improve the quality of the sheep stock (Ryder, 1983). This introduction correlates well with the calculated time of divergence, of 100-200 years ago.

By the late 19th and early 20th centuries large scale livestock movement had become rarer. However, at this time there was an increase in the movement of animals for sporting purposes. The introduction of red deer into Donegal resulted in the introduction of *Hypoderma diana*, a parasite which is only found in this area of Ireland (Sleeman, 1983). Such events may explain the introduction of British LI virus into Ireland. Similarly, in 1915 red grouse were introduced from Scotland onto Exmoor in Devon (Tapper, 1992) and this may account for the emergence of the disease in the south-west of England. However, the involvement of livestock movement or the migration of wild birds cannot be ruled out.

4.3.6. The Evolution of LI Virus

By combining the predicted dispersal pattern, interpreted from the phylogenetic trees, and the estimated times of lineage divergence, a theory of LI virus evolution can be proposed. The data imply that an ancestral LI virus was introduced into Ireland from Europe less than 600 years ago. Between 300-400 years ago the virus was transported across the Irish Sea into Wales, from where it was dispersed to Scotland, presumably to the Borders. Approximately 100-200 years ago a major dispersal event occurred with the introduction of LI virus throughout Scotland, Northern

England and Norway. More recently, about 100 years ago, LI virus from the west of Scotland was reintroduced into Ireland, where it successfully co-inhabits the tick population with Irish LI virus. The most recent dispersal event, according to the data analyzed to date, was the introduction of virus from the east of Scotland into the south-west of England, which is estimated to have occurred this century. This is supported by the fact that LI disease was first reported on moorland farms in the south-west of England in the 1930s (Reid, pers. com.).

This predicted dispersal pattern contradicts the traditional view that the ancestral LI virus was present in the forests of Britain since the end of the last ice-age and emerged as a disease of sheep when the Caledonian forest was replaced by moorland. This deforestation took centuries to complete with periods of accelerated felling for timber during times of war. Grazing pressures prevented the forests re-establishing while heather, which was a component of the ground cover in the original forests, thrived creating today's moorland.

If LI virus or the ancestral virus was present in the British Isles since the end of the last ice-age then the virus would have spread northwards with *I. ricinus*, following the retreating ice-cap, from either the south-west of England or Europe. Therefore phylogenetic analysis of LI viruses would be expected to show a general northwards dispersal pattern with different lineages of virus being amplified in particular areas when sheep farming was introduced. However, this is clearly not the case. Moreover, phylogenetic analysis in this and a previous study (Zanotto *et al.*, 1996b) estimate that LI virus evolution has only occurred within the last millennium.

The involvement of humans and in particular the movement of livestock, have been implicated in the dispersal of LI virus, but this can never be absolutely demonstrated. However, historical records provide information of a large number of events which could have resulted in the movement of the virus. In particular, the correlation between the major dispersal of sheep from the Borders of Scotland to the rest of Scotland, Northern England and Norway and the estimated time of lineage divergence strongly implies that livestock movement was involved in the dispersal of LI virus.

The predicted dispersal events described above fit the data available at present. However, the non-random nature of the collection of these viruses may have led to a historically important population of LI virus being excluded from the analysis. It is important to note that only one LI virus from Wales has been available to study. Considering the seemingly important role of this single virus in the dispersal of LI virus it is vital to understand more about it, especially to determine if LI/I virus truly represents the Welsh LI virus population. Furthermore, only a single LI virus from the Borders of Scotland has been studied. The study of additional LI viruses can only extend the analysis reported here and refine the proposed dispersal pattern of LI virus described above.

Amino Acid	Nucleotide									
	DEV4	INV1	INV6	INV14	IRE3	LI/261	LI/31	LI/369	LI/917	LI/A
DEV4	-	98.99	99.06	97.92	97.85	96.64	96.44	97.72	97.51	99.66
INV1	99.40	-	98.86	97.72	97.65	96.51	96.17	97.51	97.18	98.93
INV6	98.99	99.19	-	98.05	98.12	96.91	96.30	97.98	97.51	98.99
INV14	98.99	99.19	99.19	-	97.65	96.57	95.90	97.45	97.04	98.12
IRE3	98.79	98.99	98.99	99.80	-	97.18	96.37	98.32	97.38	97.92
LI/261	98.59	98.79	99.19	99.19	98.99	-	95.36	96.98	96.57	96.71
LI/31	98.39	98.59	98.19	98.59	98.79	98.19	-	95.83	96.04	96.51
LI/369	98.79	98.99	98.99	99.80	99.60	98.99	98.39	-	97.04	97.85
LI/917	98.39	98.59	98.99	98.99	98.79	98.99	98.39	98.79	-	97.58
LI/A	99.60	99.80	99.40	99.40	99.19	98.99	98.79	99.19	98.79	-
LI/G	98.59	98.79	98.79	99.19	98.99	98.79	98.19	98.99	98.59	98.99
LI/I	97.38	97.58	97.78	97.98	97.78	97.78	97.18	97.78	97.58	97.78
LI/K	99.19	99.40	99.40	99.80	99.60	99.40	98.79	99.60	99.19	99.60
LI/NOR	98.99	99.19	99.19	99.60	99.40	99.19	98.59	99.40	98.99	99.40
MA54	95.57	95.77	95.97	96.17	96.37	95.77	95.57	96.37	95.57	95.97
PEN3	98.79	98.99	98.99	99.40	99.19	98.99	98.39	99.19	98.79	99.19
PEN6	98.39	98.59	98.99	98.99	98.79	98.59	97.98	98.79	98.39	98.79
SB/526	98.19	98.39	97.98	98.39	98.59	97.98	99.40	98.19	98.19	98.59
THO1	99.19	99.40	99.40	99.80	99.60	99.40	98.79	99.60	99.19	99.60
THO2	99.19	99.40	99.40	99.80	99.60	99.40	98.79	99.60	99.19	99.60

Amino Acid	Nucleotide									
	LI/G	LI/I	LI/K	LI/NOR	MA54	PEN3	PEN6	SB/526	THO1	THO2
DEV4	97.11	93.28	98.86	97.51	91.87	97.51	97.25	96.17	99.13	99.06
INV1	96.91	93.28	98.66	97.25	91.80	97.38	97.11	95.90	98.93	98.86
INV6	97.38	93.41	98.86	97.51	92.14	97.65	97.51	96.04	99.13	99.06
INV14	97.31	93.21	97.92	97.45	91.94	97.18	96.91	95.90	98.12	98.05
IRE3	97.78	93.75	98.05	97.45	92.27	97.51	97.38	96.10	98.05	97.98
LI/261	96.71	92.68	96.71	96.64	91.53	96.57	96.44	94.96	96.84	96.77
LI/31	95.30	93.08	96.10	96.17	91.80	95.77	95.63	99.19	96.37	96.30
LI/369	97.72	93.75	97.92	97.51	92.54	97.65	97.51	95.57	97.92	97.85
LI/917	96.77	93.35	97.18	97.51	92.00	97.04	96.77	95.77	97.45	97.38
LI/A	97.31	93.21	98.79	97.72	92.07	97.45	97.18	96.37	99.06	98.99
LI/G	-	93.21	97.04	96.84	91.87	96.91	96.77	95.30	97.45	97.38
LI/I	97.98	-	93.62	93.55	93.28	93.41	93.28	92.88	93.35	93.28
LI/K	99.40	98.19	-	97.45	92.07	97.45	97.18	95.70	99.06	98.99
LI/NOR	99.19	97.98	99.80	-	92.61	97.25	96.98	95.90	97.58	97.51
MA54	96.17	96.17	96.37	96.17	-	91.73	91.73	91.53	91.94	91.87
PEN3	98.99	97.78	99.60	99.40	95.97	-	99.73	95.50	97.72	97.65
PEN6	98.59	97.38	99.19	98.99	96.17	99.60	-	95.36	97.45	97.38
SB/526	97.98	96.98	98.59	98.39	95.36	98.19	97.78	-	95.97	96.04
THO1	99.40	98.19	100.0	99.80	96.37	99.60	99.19	98.59	-	99.93
THO2	99.40	98.19	100.0	99.80	96.37	99.60	99.19	98.59	100.0	-

Table 4.1. Pairwise percentage similarities of the complete E gene sequence of LI viruses. The percentage nucleotide sequence similarities are recorded in the top half of the tables and the deduced amino acid sequence similarities are recorded in the bottom half.

Viruses	Estimated Synonymous Substitution Rate
DEV4 & LI/A	-3.20×10^{-4}
INV6 & LI/K	7.17×10^{-4}
LI/917 & LI/261	-1.00×10^{-4}
LI/G & IRE3	8.00×10^{-4}
LI/I & MA54	5.17×10^{-4}
LI/31 & SB/526	4.30×10^{-4}
PEN3 & INV14	-8.00×10^{-4}
PEN6 & LI/369	2.18×10^{-3}
THO1 & THO2	8.67×10^{-4}

Table 4.2. Estimated synonymous substitution rates calculated for different LI virus pairs using the method proposed by Li *et al.* (1988). Values were calculated using SSE virus as the outgroup and the synonymous pairwise genetic distances calculated using the Nei-Gojobori (1986) method available in the MEGA software package (Kumar *et al.*, 1993).

	INV1	INV6	INV14	IRE3	LI/31	LI/261	LI/369	LI/917	LI/A	LI/G
DEV4	44	33	83	95	178	143	104	97	18	123
INV1		51	102	114	201	159	123	123	61	142
INV6			84	90	185	144	99	111	50	117
INV14				123	211	158	135	132	88	124
IRE3					202	136	101	123	106	109
LI/31						236	225	204	189	243
LI/261							148	159	154	151
LI/369								143	112	115
LI/917									108	145
LI/A										127

	LI/I	LI/K	LI/NOR	MA54	PEN3	PEN6	SB/526	THO1	THO2
DEV4	317	48	107	366	104	111	170	34	34
INV1	326	67	130	380	120	127	194	52	52
INV6	318	56	115	356	105	112	177	43	42
INV14	335	104	122	376	132	139	189	92	92
IRE3	308	106	131	369	124	124	194	104	104
LI/31	350	210	201	397	220	219	74	194	194
LI/261	368	161	159	398	160	159	237	152	153
LI/369	310	115	130	354	120	120	218	113	113
LI/917	320	134	112	362	133	140	197	119	119
LI/A	337	65	112	369	122	128	174	51	51
LI/G	340	147	152	386	145	145	221	125	125
LI/I		319	318	295	323	322	340	335	336
LI/K			131	377	128	135	210	57	57
LI/NOR				336	134	140	194	123	123
MA54					390	390	393	385	386
PEN3						20	212	113	113
PEN6							212	119	120
SB/526								193	187
THO1									13

Table 4.3. Estimates of the date of lineage divergence (years from present) for LI viruses. Values were calculated using the synonymous pairwise genetic distances calculated using the Nei-Gojobori (1986) method available in the MEGA software package (Kumar *et al.*, 1993) and the mean synonymous substitution rate (4.76×10^{-4} substitutions/site/year) as estimated using the method of Li *et al.* (1988).

Amino acid	Nucleotide												
	INV6	LI/31	LI/I	MA54	SSE	WTBE	TSE	FETBE	Vs	OMSK	LGT	KFD	POW
INV6	-	99.30	93.41	92.14	89.65	87.10	83.74	82.33	82.16	79.23	74.19	73.05	69.02
LI/31	98.19	-	93.08	91.80	89.99	86.69	83.54	82.19	81.59	79.03	78.86	73.19	69.02
LI/I	97.78	97.18	-	93.28	90.52	86.69	82.66	82.33	83.13	78.76	74.19	72.58	68.88
MA54	95.97	95.57	96.17	-	90.86	87.30	83.40	82.46	83.13	79.50	74.53	73.05	69.49
SSE	95.77	96.17	95.77	95.97	-	86.56	84.14	81.59	82.46	80.04	74.06	75.25	69.09
WTBE	93.75	93.95	93.75	93.75	94.96	-	84.61	84.14	84.07	81.79	75.27	73.86	69.15
TSE	93.55	93.55	93.15	93.55	94.15	95.97	-	82.06	81.38	80.58	75.67	72.51	69.49
FETBE	90.93	91.13	91.33	92.74	92.74	95.57	93.74	-	84.95	80.85	75.07	72.91	70.09
Vs	91.13	91.33	91.53	92.34	92.54	94.96	92.34	95.77	-	80.44	75.43	72.25	69.76
OMSK	89.72	89.72	89.72	90.32	90.52	93.15	91.73	93.75	91.94	-	74.66	71.71	69.76
LGT	84.68	84.68	84.27	86.09	86.09	87.50	86.09	88.11	87.10	86.90	-	72.45	69.76
KFD	79.84	80.24	79.84	80.65	81.45	81.25	80.65	80.65	80.65	80.65	79.74	-	69.56
POW	76.82	77.42	77.42	77.82	78.02	78.23	77.02	78.02	77.62	77.62	78.04	77.42	-

Table 4.4. Pairwise percentage nucleotide and deduced amino acid similarities for the complete E gene sequences of selected LI viruses and other members of the TBE virus serocomplex. The percentage nucleotide sequence similarities are recorded in the top half of the table and the deduced amino acid sequence similarities are recorded in the bottom half. The Neudoerfl and Sofjin strains of WTBE and FETBE respectively were used in the analysis (Mandl *et al.*, 1988; Yamshchikov & Pletnev, 1988).

Fig. 4.1. Illustration of the heterogeneous sequence of the IRE4 isolate. Autoradiograph of the nucleotide sequence of a) IRE4 and b) IRE3 virus. The nucleotide sequences were determined in both directions. The marked sequences encode the tripeptide genetic marker; NPH for IRE3, NPH and GPR for IRE4. The single amino acid code has been used.

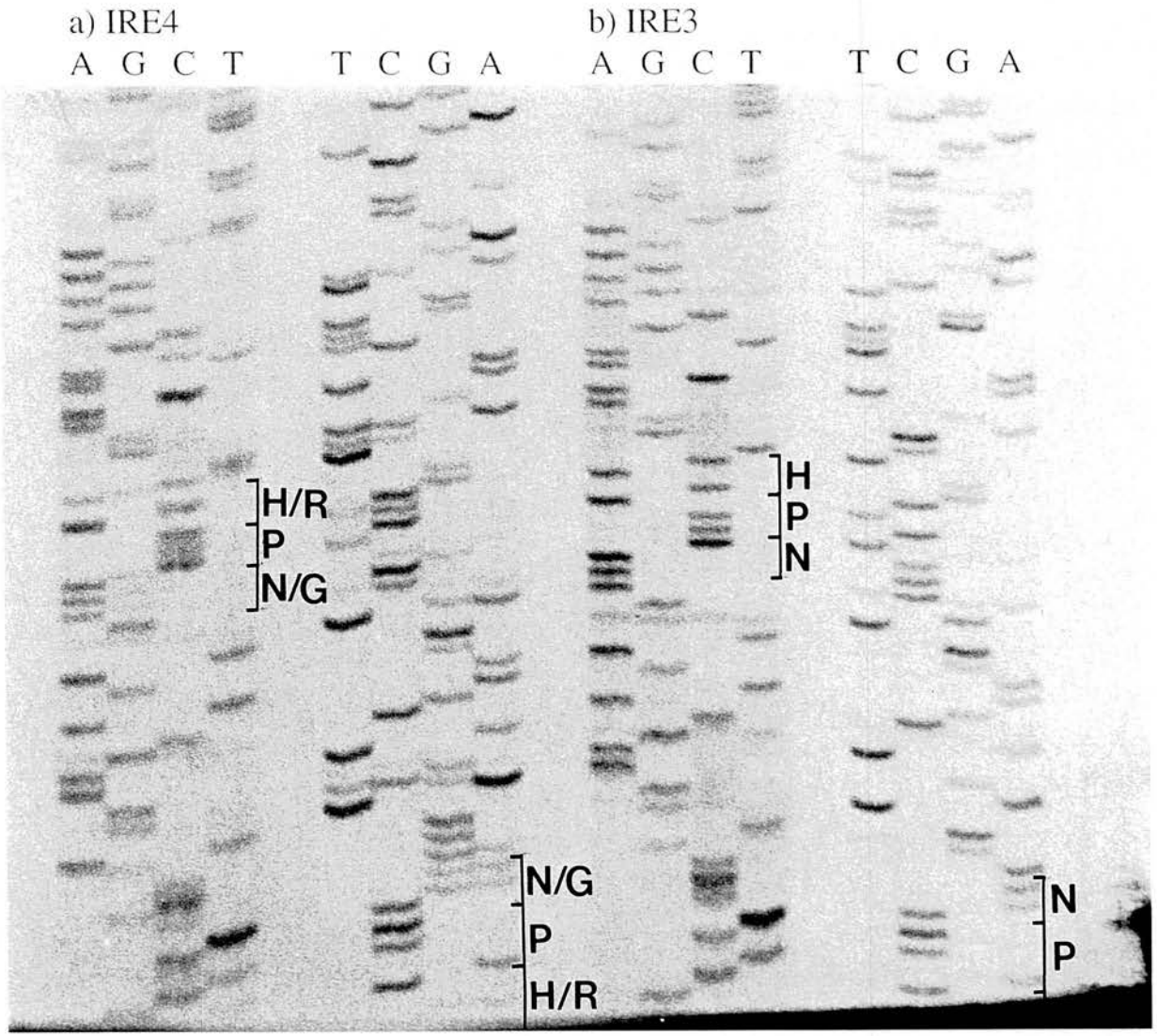

a) IRE4
A G C T
T C G A
H/R
P
N/G
N/G
P
H/R
b) IRE3
A G C T
T C G A
H
P
N
N
P

Fig. 4.2. Maximum likelihood phylogenetic tree of the E gene RGF of LI viruses and closely related TBE serocomplex flaviviruses. Branch lengths are drawn to scale and all nodes supported by more than 75% bootstrap replications are indicated. The tree is rooted with the sequence of FETBE, Sofjin strain (Yamshchikov & Pletnev, 1988) and the Neudoerfl strain of WTBE was incorporated into the study (Mandl *et al.*, 1988). Although the Welsh virus LI/I is shown as being more closely related to the Irish viruses than the main cluster of British sequences, most of the bootstrap replications (85%) place the virus closer to the British group (circled node).

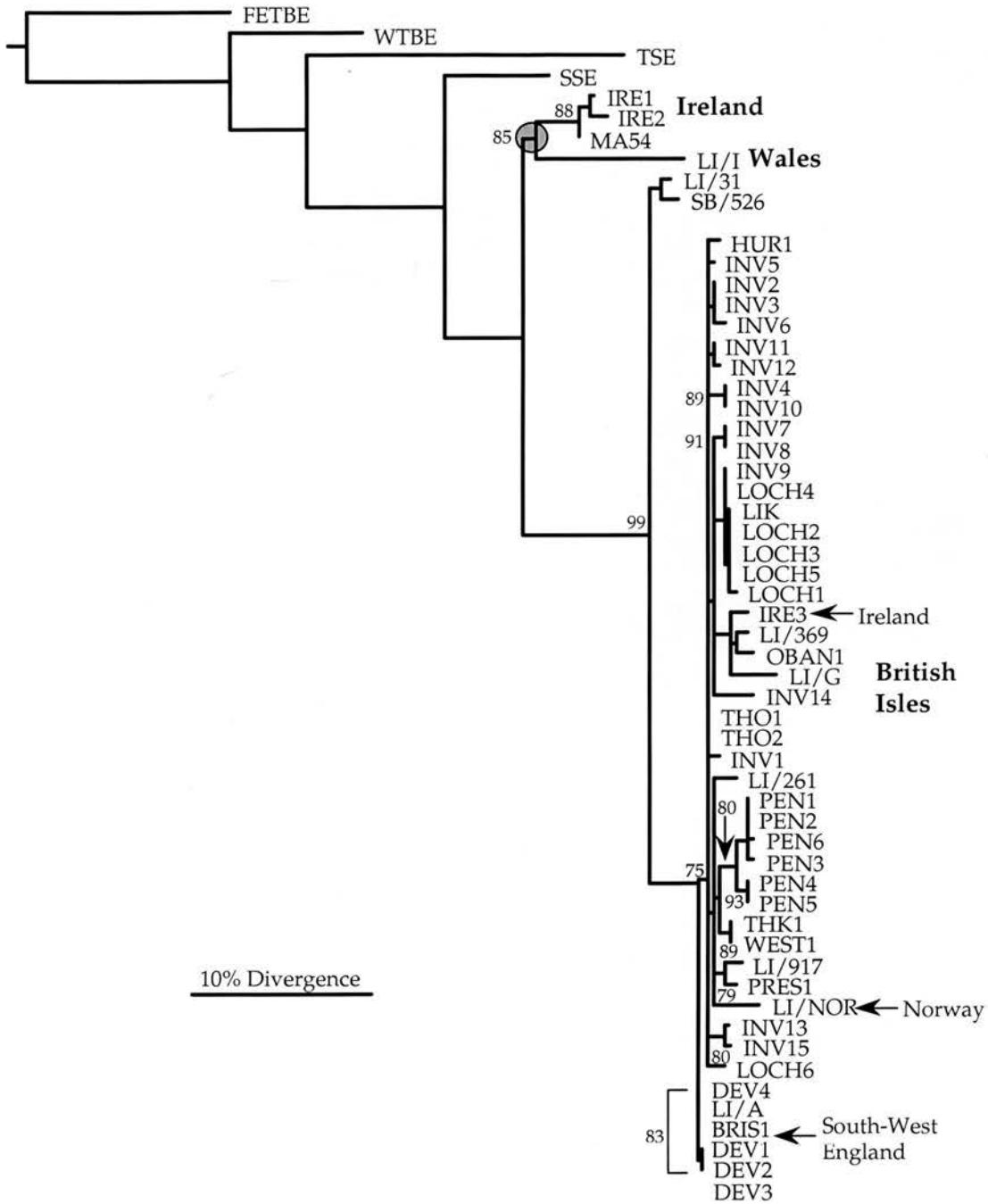

FETBE
WTBE
TSE
SSE
IRE1
IRE2
MA54
Ireland
LI/I
Wales
LI/31
SB/526
HUR1
INV5
INV2
INV3
INV6
INV11
INV12
INV4
INV10
INV7
INV8
INV9
LOCH4
LIK
LOCH2
LOCH3
LOCH5
LOCH1
IRE3
Ireland
LI/369
OBAN1
LI/G
INV14
British Isles
THO1
THO2
INV1
LI/261
PEN1
PEN2
PEN6
PEN3
PEN4
PEN5
THK1
WEST1
LI/917
PRES1
LI/NOR
Norway
INV13
INV15
LOCH6
DEV4
LI/A
BRIS1
DEV1
DEV2
DEV3
South-West England
88
85
99
89
91
80
75
93
89
79
80
83
10% Divergence

Fig. 4.2.

Fig. 4.3. Maximum likelihood phylogenetic tree of the complete E gene of LI viruses and closely related TBE serocomplex flaviviruses. Branch lengths are drawn to scale and all nodes supported by more than 75% bootstrap replications are indicated. The tree is rooted with the sequence of FETBE, Sofjin strain (Yamshchikov & Pletnev, 1988) and the Neudoerfl strain of WTBE was incorporated into the study (Mandl *et al.*, 1988).

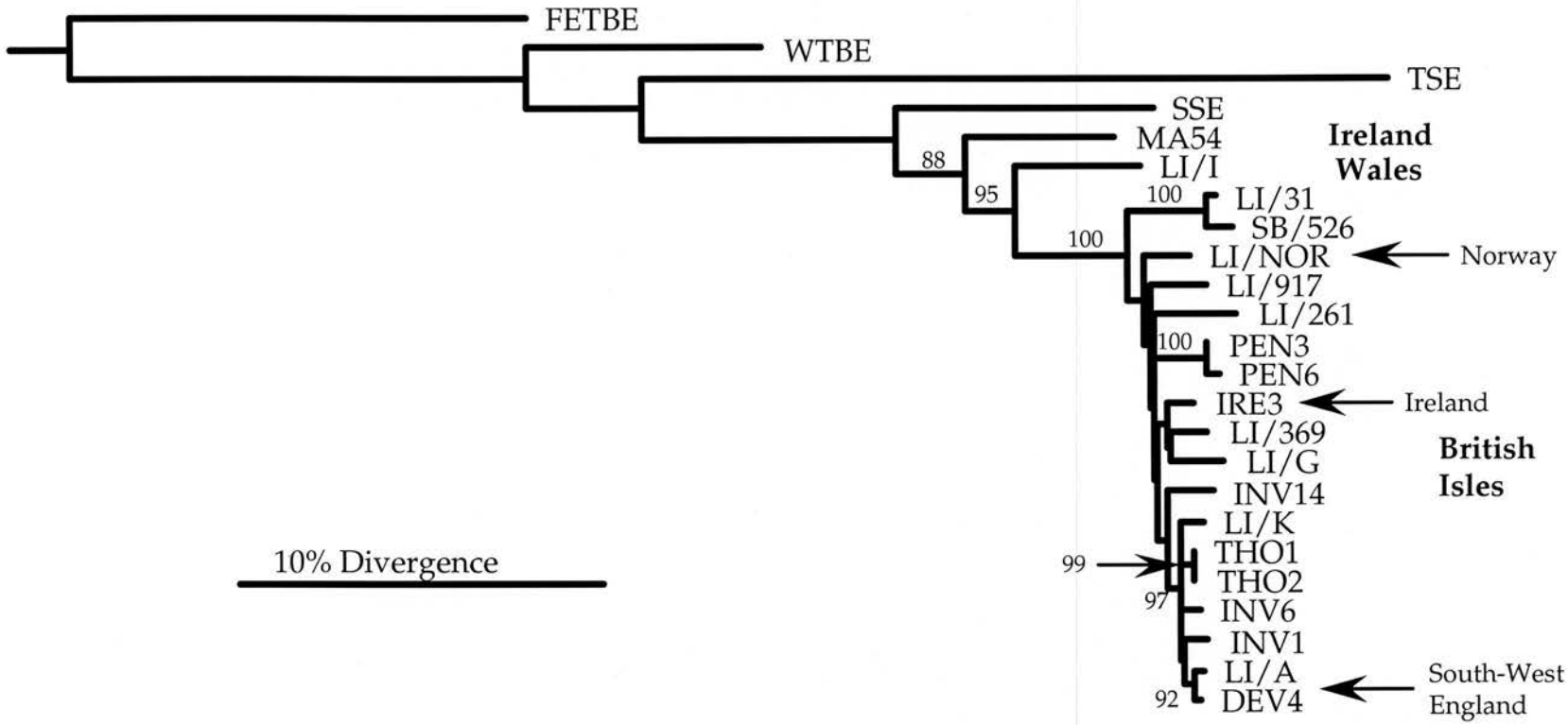

FETBE
WTBE
TSE
SSE
MA54
LI/I
88
95
100
LI/31
SB/526
100
LI/NOR
Norway
LI/917
LI/261
100
PEN3
PEN6
IRE3
Ireland
LI/369
LI/G
INV14
LI/K
THO1
THO2
INV6
INV1
LI/A
DEV4
Ireland
Wales
British
Isles
99
97
92
South-West
England
10% Divergence

Fig. 4.3.

Fig. 4.4. Predicted dispersal pattern of LI virus. 1) initial introduction of the ancestral virus into Ireland. 2) introduction of Irish LI virus into Wales. 3) movement of virus into Scotland. 4) major dispersal of LI virus throughout Scotland and the north of England. 5) introduction of LI virus into Norway. 6) reintroduction of LI virus into Ireland from the west of Scotland. 7) introduction of LI virus into the south-west of England from the east of Scotland.

Fig. 4.4.

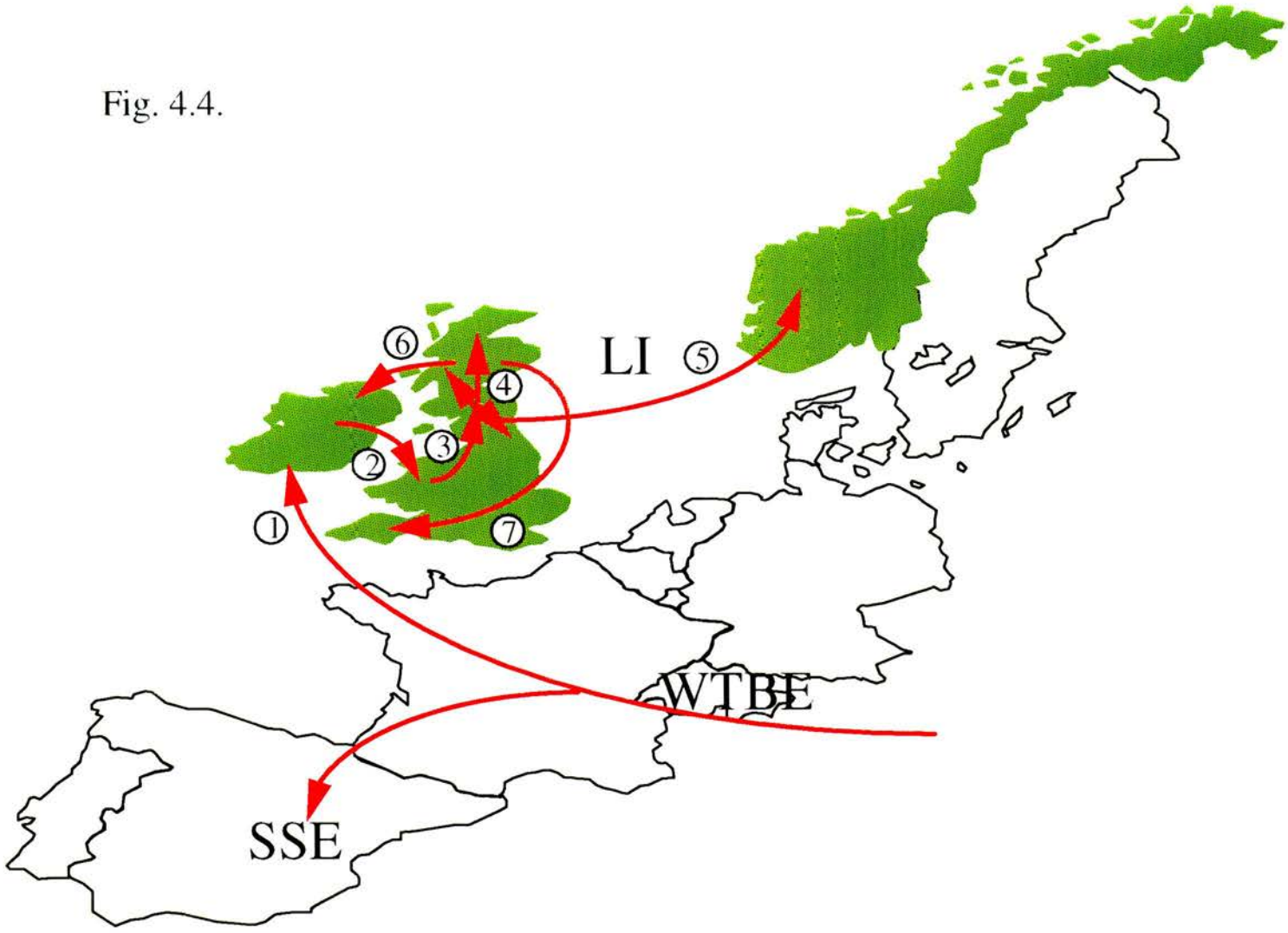


Fig. 4.5. Neighbour-joining bootstrap tree (1000 replications) with only those branches in more than 75% of replicates shown as bifurcating (all other branches with lower bootstrap support are left unresolved). The distances between the sequences were estimated under the same model of RNA substitution as in the maximum likelihood analysis. The tree is rooted using FETBE virus, Sofjin strain (Yamshchikov & Pletnev, 1988). The horizontal branch lengths are not drawn to scale.

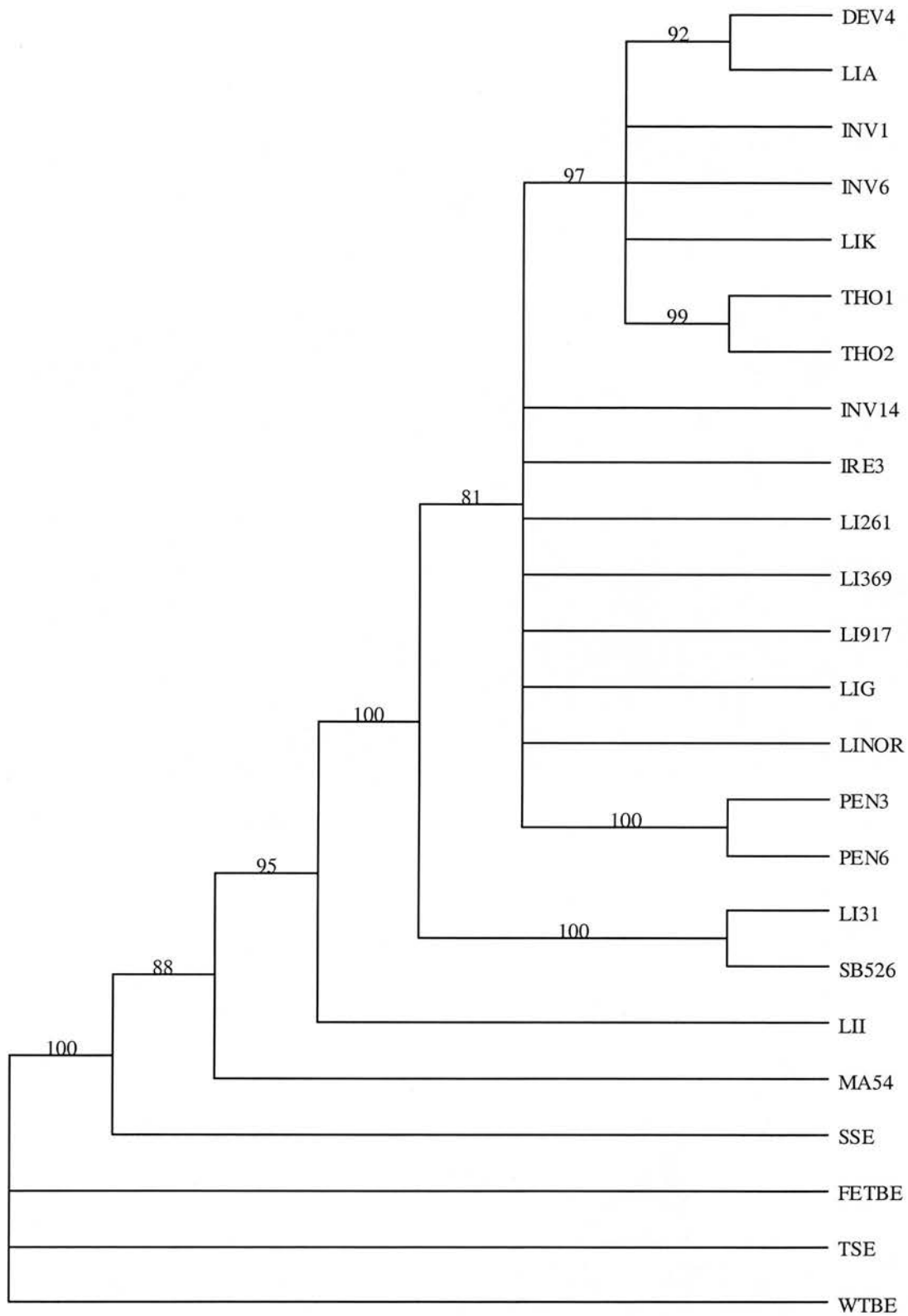

DEV4
LIA
INV1
INV6
LIK
THO1
THO2
INV14
IRE3
LI261
LI369
LI917
LIG
LINOR
PEN3
PEN6
LI31
SB526
LII
MA54
SSE
FETBE
TSE
WTBE
92
97
99
81
100
100
95
100
88
100

Fig. 4.5.

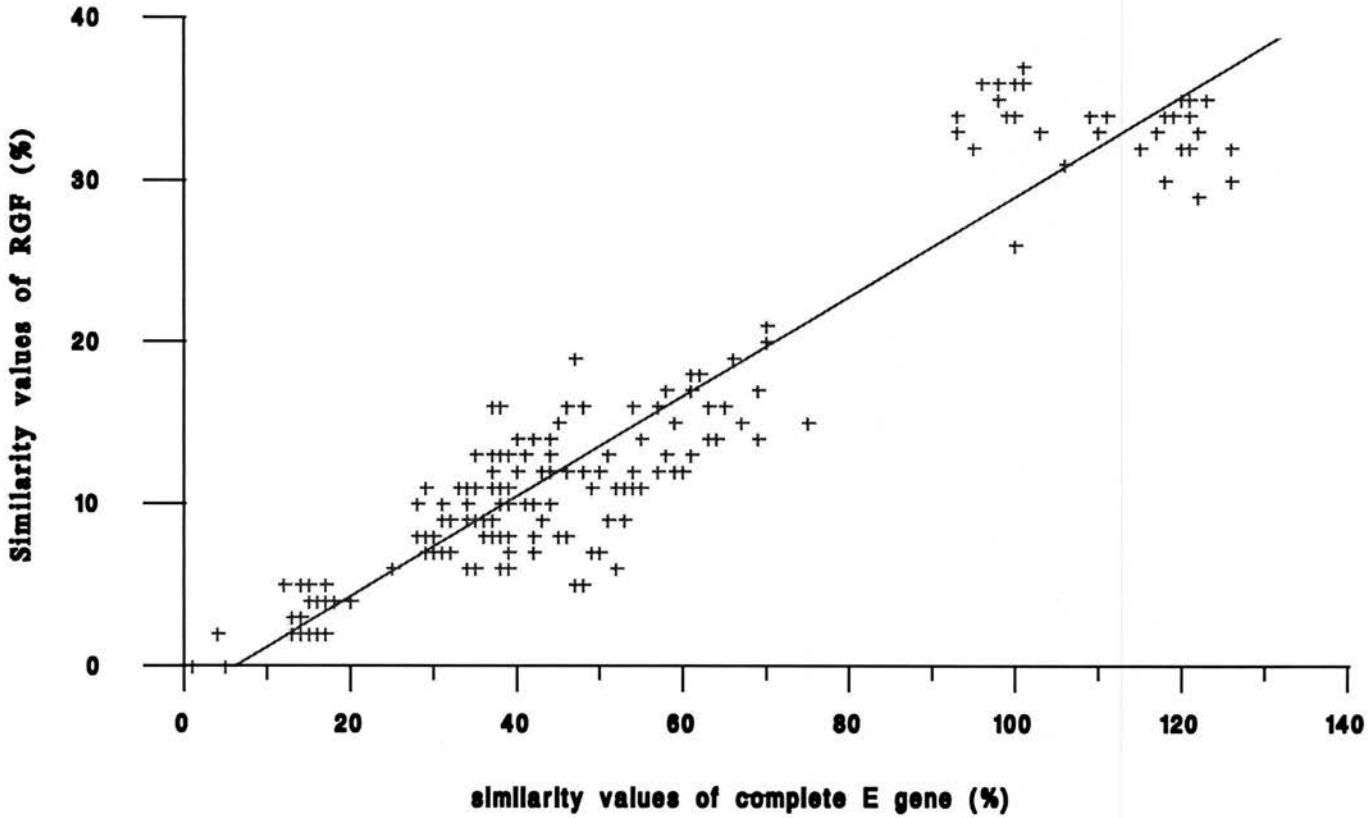


Fig. 4.6. Comparison of the pairwise percentage nucleotide sequence similarities of LI viruses generated using the complete E gene and RGF sequence data. There is strong correlation between the two homology data sets ($r = 0.89$) and the equation of the line of best fit is; $y = -1.93 + 0.31x$.

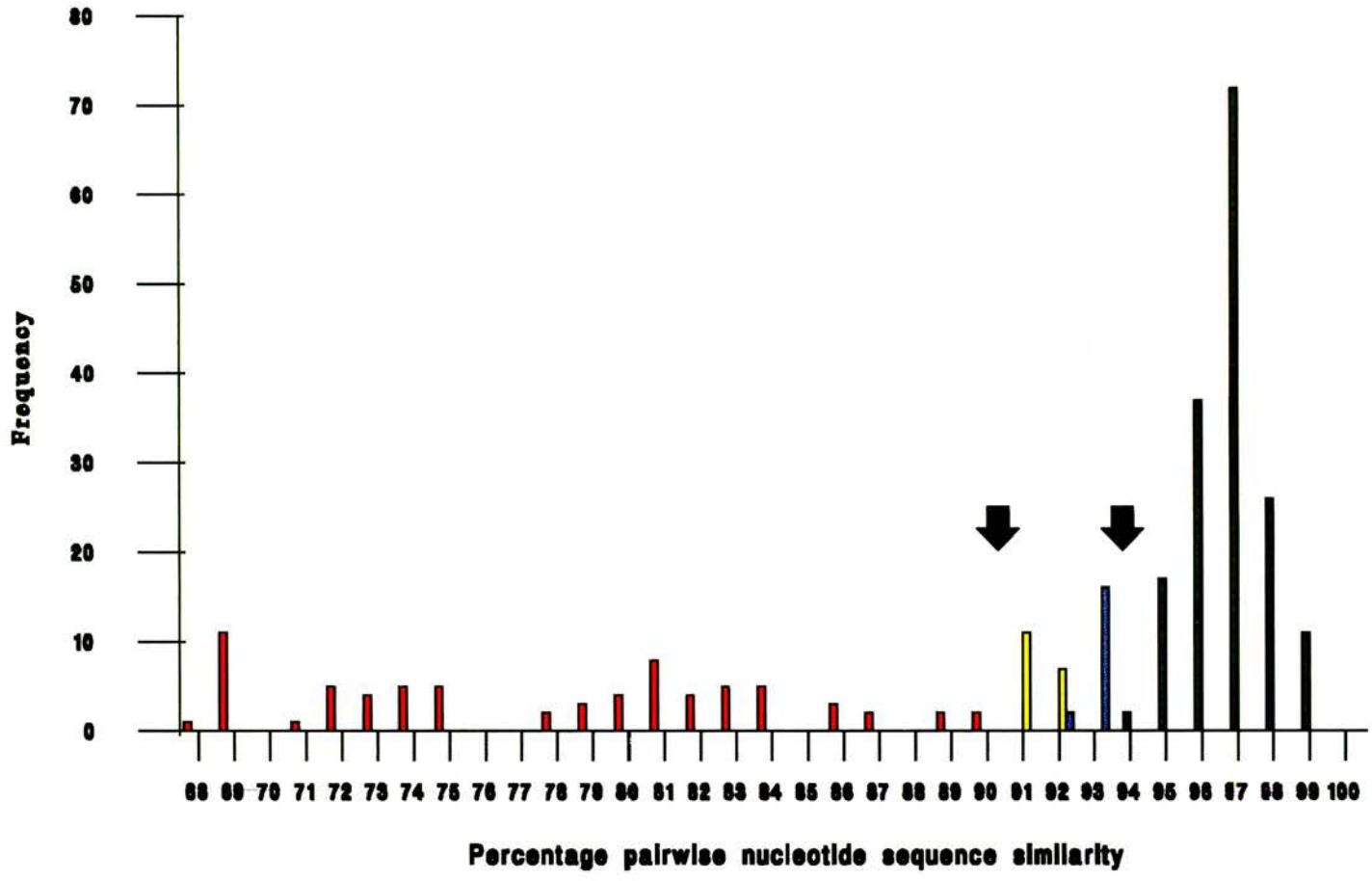


Fig. 4.7. Frequency distribution of pairwise nucleotide sequence similarities for members of the TBE virus serocomplex. Arrows represent the cut-off margin between intraspecies and interspecies variation. ■ denotes interspecies variation, ■ denotes intraspecies variation, and □ and ■ denote variation between British LI viruses and Irish and Welsh viruses respectively.

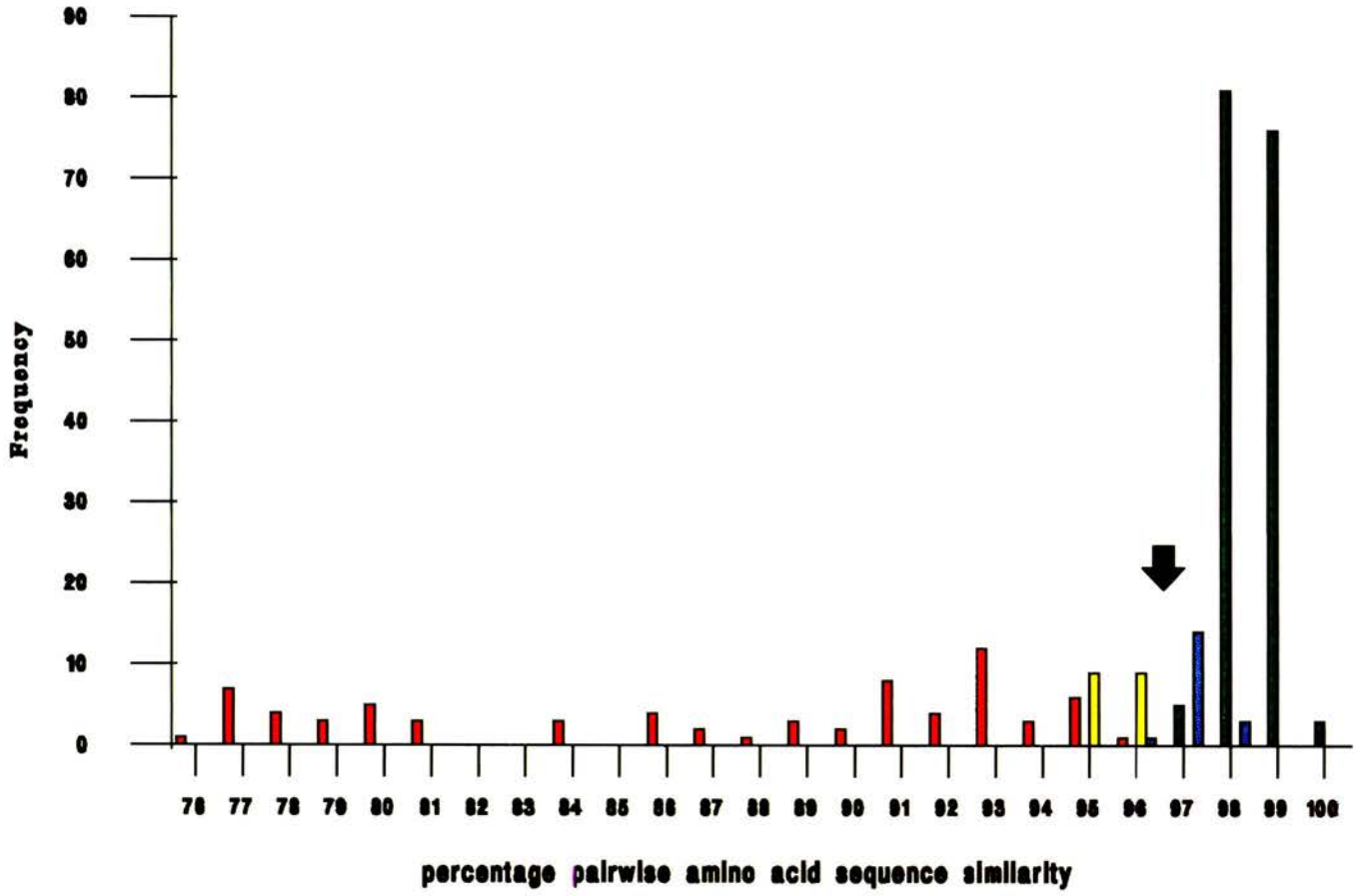


Fig. 4.8. Frequency distribution of pairwise amino acid sequence similarities for members of the TBE virus serocomplex. Arrow represents the cut-off margin between intraspecies and interspecies variation. ■ denotes interspecies variation, ■ denotes intraspecies variation, and □ and ■ denote variation between British LI viruses and Irish and Welsh viruses respectively.

CHAPTER FIVE

Variation in the Biological Properties of Louping-ill Viruses

CONTENTS

5.1. Introduction

Investigations of the antigenic and genetic variation among LI viruses were described in the previous results chapters. A number of interesting differences were identified; including the presence of different genetic markers, the occurrence of escape variants and an association between genetic variation and geographic distribution. In this chapter the biological variation among LI viruses is investigated *in vivo* and *in vitro,* to see whether or not there is a correlation between biological characteristics and genetic variation. Initially, the cytopathogenicity of viruses was compared by plaque morphology analysis, followed by an investigation of the virulence characteristics and resulting histopathology of selected LI viruses in mice.

Mice were identified as a suitable laboratory model for LI virus infection in the 1930s, when it was also shown that rabbits, guinea pigs and rats were not suitable models (Alston & Gibson, 1931; Greig *et al.,* 1931; Hurst, 1931). The symptoms and lesions observed in mice after LI virus infection were similar to those seen in sheep (Greig *et al.*, 1931). Typical symptoms include; hyperaethesia, reflex excitability, loss of coat condition, hunched appearance, muscle spasm, fine tremor, unsteadiness, irregular breathing and paralysis, especially of the hind limbs, leading to coma and death (Alston & Gibson, 1931; Greig *et al.*, 1931; Hurst, 1931). Histopathological analysis of brain tissue revealed widespread lesions including; perivascular cuffing, glial foci and neurone degradation. Areas of the brain particularly affected included the cerebrum, cerebellum, thalamus, corpus callosum, brain stem and spinal cord (Hurst, 1931; Doherty, 1969b). Pogodina & Savinov (1964) showed that the mouse strain C57/BL was susceptible to a number of TBE serocomplex flaviviruses. It was later shown that this was also true for LI virus (Doherty, 1969b). Therefore C57/BL was the mouse strain initially chosen for use in this study.

Hussain (1990) detected variation in the virulence characteristics of LI virus for SW mice and also found a direct correlation between pathogenicity and plaque morphology. The plaque assay technique was originally developed by Dulbecco (1952) and provides a relatively quick and simple method to investigate differences between viruses. Heterogeneity in plaque morphology reflects variation in the rate of

multiplication of single virus infective units (Dulbecco, 1952; Dulbecco & Vogt, 1954). Dubes (1956) suggested that the plaque sizes of poliovirus strains were influenced by a single genetic determinant. Hussain (1990) identified considerable variation in plaque morphology between non-plaque purified LI viruses. Escape variants produced significantly smaller plaques than other viruses which bound MAbs 4.2 and 7.1. In addition viruses from Ireland produced significantly larger plaques than viruses collected in Britain.

However, the antigenic variation among LI viruses is now known to be more complex than was first thought with the identification in this study of two types of escape variants. Furthermore, two distinct populations of virus have been identified in Ireland. Therefore a study was undertaken to investigate the biological variation among a selection of LI viruses representative of the virus population identified in the British Isles. In addition to studying the biological characteristics of LI viruses an attempt was made to investigate the importance of the antigenic and genetic variation detected earlier in this study. However, it is recognized that without knowledge of the complete genome sequence, any identified associations between biological and genetic variation cannot be absolutely attributed to the genetic substitutions identified.

5.2. Results

5.2.1. Plaque Morphology

Plaque assays were carried out on PK cell monolayers for a total of nineteen LI viruses (Table 5.1) and the individual necrotic area of ten plaques was measured for each virus. The results are summarized in Table 5.2 and recorded in full in Appendix 5.1. There was a remarkable range of plaques sizes from 0.94-8.63 mm^2, illustrated in Fig. 5.1. Considerable heterogeneity was even observed in the size of plaques produced by each virus. The greatest variation was detected in LOCH2 virus for which the plaque size ranged from 2.69-8.63 mm^2.

There was a statistically significant association between plaque size and antigenic phenotype (W=10.7 P=0.005, Wald test). A statistically significant difference was detected between plaques produced by type-I and type-II escape variants. Type-II escape variants produced smaller plaques than type-I escape variants, which in turn produced smaller plaques than wild-type viruses. The difference between type-I escape variants and wild-type viruses is borderline in terms of the statistical significance. Moreover, there was a highly statistically significant association between plaque morphology and geographic distribution (W=100.4 P<0.001, Wald test). Viruses from Ireland produced significantly larger plaques than viruses from England and Scotland. In addition, English viruses tended to produce smaller plaques than Scottish viruses, although this effect was not statistically significant.

Several viruses from similar locations, which share substantial sequence similarity within the E protein, produced plaques which differed considerably in size. PEN3 and PEN6 virus, which share 99.6% amino acid sequence similarity within the E protein, produced plaques of significantly different size (P=0.028, t-test).

The Irish and British LI viruses, IRE2 and IRE3 respectively, produced plaques which did not differ significantly in size (P=0.419, t-test), although these viruses share only 96.4% amino acid sequence similarity within the E protein. However, LI/369, the LI virus most closely related genetically to IRE3 virus, sharing 99.6% amino acid sequence similarity within the E protein, produced plaques which were significantly smaller than those produced by IRE3 virus (P=0.002, t-test).

5.2.2. LI Virus Virulence in C57/BL Mice

Initially the mortality rates in mice resulting from the inoculation of ten different LI viruses (Table 5.1) was investigated in the C57/BL mouse strain. Groups of twenty 3-5 week old mice were inoculated subcutaneously with between 1.2-3.4 x 10^3 p.f.u. of virus and observed for 21 days. The mice developed symptoms which implied a neurological disease including; loss of coat condition, hunched appearance, ataxia, apathy, paralysis of hind limbs and death. The course of the disease could be slow or

swift and resulted in the death of a number of study animals between the 12 hourly observations. However, the majority of mice were euthanized *in extremis* when comatose or acutely paralyzed.

The cumulative mortalities differed considerably for different viruses as illustrated in Fig. 5.2 and recorded in Appendix 5.2. The most pathogenic viruses investigated were IRE2 and INV1 which caused 95% mortality by 13 and 15 days post inoculation (p.i.) respectively. IRE3 virus was also pathogenic resulting in 90% mortality. A cluster of viruses; DEV4, INV6, PEN3, PRES1 and SB/526, exhibited similar pathogenicity traits ranging from 65-75%. PEN6 virus was the most attenuated, killing only 5 (25%) mice, followed by LI/A virus which caused 35% mortality. The time of disease onset also differed between the viruses, for example the earliest death of an IRE2 virus inoculated mouse occurred on day 7 p.i. In contrast, mice inoculated with DEV4, LI/A and PEN6, the three most attenuated viruses, only began to show signs of disease and die from day 10 p.i.

The initial dose of each virus differed due to inaccuracies in estimating the titre of virus inoculum. However, no correlation was detected between the initial dose of virus inoculated into mice and the average survival time ($r = -0.20$).

Equal numbers of male and female mice were inoculated with each virus and therefore the effect of mouse gender on the virulence characteristics of LI viruses was investigated. The cumulative mortalities observed in male and female animals are illustrated in Fig. 5.3 and summarized in Appendix 5.2. SB/526 virus first caused mortality in male and female mice on the same day, unlike the majority of cases where inoculation with LI viruses resulted in female animals developing symptoms 1-3 days prior to males. The largest difference of 3 days was observed in mice inoculated with PEN6 and IRE3 virus. Female mice inoculated with IRE3 virus became ill on day 8 while male animals first showed signs of disease on day 11, by which time 80% of female mice had died. At the other extreme male mice inoculated with PEN3 virus showed signs of disease three days before symptoms appeared in females.

Most viruses caused similar levels of pathogenicity in male and female animals. However, higher numbers of female than male animals developed disease after

inoculation with INV6 and PRES1 virus. INV6 virus caused 90% and 40% mortality in female and male animals respectively, which is a statistically significant difference (P=0.046). In contrast, inoculation with PEN3 virus produced higher mortality in male than female C57/BL mice, resulting in 90% and 50% mortality respectively, although this is not statistically significant.

5.2.3. LI Virus Virulence in SW Mice

The brains of all C57/BL mice which died or were euthanized were collected and processed for histopathological analysis. The results will be described in more detail below. However, little evidence of neurological damage was detected in these brains. Therefore the analysis was partially repeated in SW mice to investigate the effect of mouse strain on the course of LI virus infection.

Three LI viruses were chosen for this analysis; IRE2, PEN6 and INV6 which exhibited high virulence, attenuation and intermediate pathogenicity in C57/BL mice. Groups of twenty 3-5 week old SW mice were inoculated subcutaneously with between 1.9-2.1 x 10^3 p.f.u. of virus and observed for 21 days. The symptoms resulting from LI virus inoculation were similar to those observed in C57/BL mice, although the neurological symptoms were more pronounced and associated with loss of balance and reflex excitability.

A comparison of the mortality caused by the three viruses in C57/BL and SW mice is illustrated in Fig. 5.4 and summarized in Appendix 5.3. There was a statistically significant difference in the cumulative mortalities of the two mouse strains (P<0.001). The onset of disease was 1-3 days earlier in SW mice than in C57/BL mice. IRE2 virus was the most pathogenic virus studied, while INV6 and PEN6 virus caused similar mortalities, although PEN6 virus appeared to have a longer incubation period than INV6 virus. The cumulative mortalities caused by IRE2 and INV6 virus were similar to those seen in the previous experiment with C57/BL mice, although in both cases the percentage mortality increased in SW mice. However, considerable variation was detected with PEN6 virus. In C57/BL mice

PEN6 virus caused only 25% mortality, but in SW mice 80% mortality was observed. Variation was not detected in the cumulative mortalities of male and female SW mice.

5.2.4. Histopathology in C57/BL and SW Mice

It was briefly mentioned above that LI virus induced little neuropathology in the brains of C57/BL mice. A number of brains were processed from animals which died between the 12 hourly observations and these also showed little damage resulting from infection except meningeal infiltration.

Histopathological analysis was also carried out on the brains of SW mice collected during the second mortality experiment. The brains of three mice, collected between days 6-12 p.i., were randomly selected from each mouse group and examined for lesions. Evidence of non-suppurative encephalitis was detected in the majority of sections (Fig. 5.5). Typical histopathological changes included glial foci and perivascular cuffing. Spongiform change, particularly in the pons and medulla, was observed in a number of cases. There was also evidence of meningeal infiltration in several animals as well as evidence of neuronal death, although that may have been due to anoxia caused by the euthanization technique. Lesions were distributed throughout the brain, although few lesions were detected in the cerebellum. No variation in lesion type, intensity or location was detected between the viruses, although variation was detected in the lesions found in mice inoculated with the same virus.

5.2.5. Viraemia Titres Recorded During the Course of LI Virus Infection in C57/BL Mice

In general, virus replication occurs in the lymph nodes and organs of the body before it invades and replicates in the brain. Consequently, measurement of infectivity in

the blood indicates peripheral turnover of virus and measurement of infectivity in the brain assesses neuroinvasiveness and specific replication in brain tissue. Therefore the titres of virus present in the blood and brains of experimentally infected mice were compared during the first seven days after inoculation. Groups of 20 5-7 week old C57/BL mice were inoculated subcutaneously with between 1.7-8.3 x 10^2 p.f.u. of virus and blood samples were collected from five mice in each group on days 1, 3, 5 and 7. The experiment finished on day 7 as this was the first day when disease was detected in the earlier experiment.

The average viraemia detected in C57/BL mice during this period is summarized in Table 5.3 and listed in full in Appendix 5.4. The highest viraemia, of 2.22 $\log_{10}$ p.f.u./0.2 ml was detected on day three in a mouse inoculated with IRE2 virus. LI viruses presented in descending order of average viraemia titres attained were as follows; IRE3, IRE2, PRES1, LI/A, INV1, PEN6, PEN3, INV6, SB/526 and DEV4.

Viraemia was only detected in mice inoculated with PEN3 and PEN6 virus during day 1 p.i. and only on the third day in mice inoculated with DEV4 virus. The majority of viruses produced detectable viraemia during the first three days p.i., although viraemia was also detected on day 5 in mice inoculated with IRE3 and PRES1 virus. No viraemia was detected on day 7 p.i. Therefore by comparing these results with the mortality data it appears that viraemia disappeared between 1-7 days prior to the first incidence of death. However, this time lag may have been exacerbated by the different initial doses of virus administered in this and the mortality experiment and the fact that C57/BL mice in this experiment were older than those in the first mortality experiment.

The viraemia titres differed considerably for each virus. However, there was no correlation between the initial dose of virus inoculated into mice and the maximum virus titre ($r = 0.03$). There was also only a weak correlation between the maximum viraemia titre detected and the average survival time of mice from the mortality experiment ($r = -0.33$).

The levels of viraemia detected in the five mice analyzed on each day from each group also differed to a high degree, as illustrated by the large standard error values (Table 5.3). Male and female C57/BL mice were used in this experiment but

variation in the virus titres detected in mice of different genders was not investigated. However, this may partially account for the large range of virus titres detected. Moreover, on the days when virus was detected in blood samples 47% of mice inoculated with the same viruses had undetectable viraemia (Appendix 5.4), implying that the virus failed to replicate significantly in many animals. However, there was only a modest correlation between the percentage mortality and the percentage of mice with detectable viraemia ($r = 0.52$) suggesting that this is not the only factor involved in the variation in mortality caused by different LI viruses.

5.2.6. Virus titres detected in the brains of C57/BL and SW Mice

In addition to measuring viraemia detected in C57/BL mice during the first 7 days of infection the virus titre found in the brain was also investigated in the same mice. The analysis was extended by measuring the titre of virus detected in the brains of SW mice on day 6 p.i. Table 5.4 summarizes the titres of virus detected in the brains of C57/BL and SW mice and the results are recorded in full in Appendix 5.5. Virus was only detected in C57/BL mice inoculated with IRE2, IRE3 and PRES1 virus on days 5 and 7 p.i., and average titres ranged from 0.36-1.33 $\log_{10}$ p.f.u./0.2 gm. There was a strong correlation between the maximum viraemia level measured in the blood and that detected in the brain ($r = 0.83$). In addition, IRE3 and PRES1 virus were also detected in the blood for the longest time period.

On day 5 three mice had detectable virus titres in their brain tissue. Two of these mice had no detectable viraemia at this time, although the mouse inoculated with PRES1 virus had a low viraemia titre. Signs of disease were first observed in C57/BL mice inoculated with IRE2, IRE3 and PRES1 virus on days 7-9 which was 2-4 days after virus was first detected in the brains of mice in these groups. However, this time lag may again have been exacerbated by differences in the initial viral dose inoculated into mice and the age of mice in the two experiments.

In contrast, all SW mice inoculated with IRE2, INV6 and PEN6 virus had detectable brain virus titres on day 6 p.i. These titres were higher than those detected

in C57/BL mice, the average titres ranging from 4.31-7.25 $\log_{10}$ p.f.u./0.2 gm. However, this may be due to higher initial doses of virus being administered to SW mice than to C57/BL mice.

The same initial dose of each virus was inoculated into SW mice involved in this and the mortality experiment and therefore the results are compatible. There was a very strong negative correlation between the virus titres detected in the brains of mice and the average survival time of that group ($r = -1$), although the significance of this is limited due to the small number of samples involved.

5.3. Discussion

5.3.1. Biological Variation Among LI Viruses

Obvious differences in the biological properties of LI viruses were identified in this study. Considerable variation was detected in the plaque sizes produced by the viruses, with viruses from Ireland producing the largest plaques and type-II escape variants producing the smallest plaques. Furthermore, extreme variation was observed in the cumulative mortalities of mice, ranging from almost complete mortality to 75% survival. Such diversity in the biological properties of LI viruses has previously been reported. Gao *et al.* (1994) recorded between 10-90% mortality of SW mice after infection with a selection of LI viruses. Hussain (1990) identified three groups of viruses according to plaque morphology which was directly correlated to mouse pathogenicity. In addition, the magnitude and duration of virus titres detected in blood and brain tissues varied to a large degree between viruses.

Two viruses collected in Ireland, IRE2 and IRE3, were included in both the *in vivo* and *in vitro* analyses. These viruses were consistently among the most pathogenic viruses investigated. In addition, C57/BL mice inoculated with IRE2 or IRE3 virus produced the highest virus titres in the brain and blood, albeit much lower than observed in SW mice. It is intriguing that two viruses with such similar biological properties have such diverse sequences, sharing 96.4% amino acid sequence

similarity within their E proteins. Within the viruses included in this study IRE3 virus is most closely related to LI/369 virus, sharing 99.6% amino acid sequence similarity within the E protein. However, the cytopathogenic effects of LI/369 and IRE3 virus are significantly different. Irish LI virus, represented by IRE2, was shown to be one of the most pathogenic LI viruses in this and previous studies (Hussain, 1990; Gao, 1995). It appears that Irish LI virus and British LI virus, represented by IRE3, are maintained within the same tick population in Ireland and therefore British LI viruses exhibiting more pathogenic properties may have been selected to compete with Irish LI virus. However, little is known about the epizootiology of these viruses in Ireland and further research would be required to investigate this theory.

The histopathology of SW mouse brains caused by infection with three LI viruses, which exhibited diverse virulence characteristics, did not differ significantly. However, variation was detected within groups of mice inoculated with the same virus. In contrast, three LI viruses; LI/I, LI/31 and MA54, were observed to cause distinct pathological effects in the brains of SW mice in a previous experiment (Hussain, 1990). However, only three viruses were investigated in this and the earlier study and the small sample size may account for the contradictory results. Variation in the histopathology of mice within a group inoculated with one virus was not detected in the earlier study. This may result from the fact that in the previous study mouse brains for analysis were collected on the same day, while in this study the brains analyzed were collected over a seven day period.

Variation was reported in the magnitude and duration of viraemia titres detected between and within groups of mice inoculated with different LI viruses. IRE2 and IRE3 virus produced the highest viraemia in C57/BL mice. Moreover, IRE3 and PRES1 virus also produced viraemia of longest duration. In a previous study of C57/BL mice, viraemia was only detected on days 1 and 2 p.i. with LI/31 virus (Doherty, 1969b).

There was a strong correlation between the magnitude of viraemia detected in the blood of C57/BL mice and the virus titre detected in the brains. A similar situation was observed in sheep. In animals which survived infection the antibody response was associated with decreasing blood titres of virus. Therefore, although virus

reached the brain and caused lesions these were not severe and the virus failed to cause clinical disease (Doherty & Reid, 1971a, b). However, a sample of mice which survived challenge in this study had no detectable lesions in their brains.

The lack of detectable lesions in the brains of C57/BL mice implies that encephalitis, normally seen in LI virus infections, did not occur. Therefore it could be argued that LI virus did not cause the mortality observed in C57/BL mice. HI antibody titres were measured in all surviving mice but few had seroconverted. However, two facts support the assumption that mortality resulted from the inoculation of LI virus. Firstly, all non-infected control mice remained healthy. Secondly, the cumulative mortalities varied between groups of mice in a manner similar to those observed in a previous study. Gao *et al.* (1994) reported 90% mortality in SW mice after inoculation with MA54 virus, 60% mortality with LI/917 virus and 20% mortality caused by LI/A virus. These results are correlated with those obtained here with IRE2, PRES1 and LI/A virus respectively. It is interesting to note that Doherty (1969b) failed to detect lesions in the brains of a quarter of C57/BL mice which developed neurological symptoms after inoculation with LI virus.

The failure to detect virus in the brains of C57/BL mice was surprising but may account for the lack of lesions detected by histopathological analysis. Neuronal necrosis is associated with the cytopathological effect of LI virus replication (Doherty & Reid, 1971a, b). The lack of virus and lesions in the brains of C57/BL mice implies that virus may affect other organs. Hurst (1931) analyzed additional organs of mice infected with LI virus but only found congestion of the lungs and distension of the bladder in a few individuals. The occurrence of neurological symptoms in C57/BL mice in this study implies that LI virus invaded and damaged the spinal cord, hence the lack of detectable lesions in the brain. This was not investigated further due to constraints on time. The lack of detectable virus titres in the brains of C57/BL mice contradicts a previous study, in which virus was consistently detected throughout the brain from day 4 to day 8 post intraperitoneal inoculation (Doherty, 1969b). However, higher doses (1.5-5.0 x 10^5 p.f.u. compared with 1.7 x 10^2 and 3.4 x 10^3 p.f.u.) were inoculated in the previous study. The initial dose of virus has

previously been shown to be important in determining the outcome of flavivirus infections (Seamer & Randles, 1967) and according to Brinton & Nathanson (1981) large viral doses can overcome the genetic resistance of mice. In addition the C57/BL mice were an average 3 weeks older than those used in the previous study (Doherty, 1969b) and the susceptibility of mice to flavivirus infection is age dependent (Sabin, 1952).

5.3.2. Association Between Plaque Morphology and Mouse Pathogenicity

The virus IRE2, which caused the highest mortality in C57/BL mice, also produced the largest plaques in cell culture. However, only a weak correlation was found between the average plaque size produced by viruses and the average mouse survival time ($r = -0.37$). Moreover, of the ten viruses included in the *in vivo* study INV1 virus produced the smallest plaques, although it was as pathogenic as IRE2 virus.

Similar results have been recorded for other flaviviruses (Barrett & Gould, 1986; Miller & Adkins, 1988; Pletnev *et al.*, 1993; Holzmann *et al.*, 1997). YF virus strain 17D-E contained virus clones which did not exhibit a correlation between plaque morphology and mouse pathogenicity, although clones from strain 17D-SA did exhibit a correlation (Liprandi, 1981). Similarly, Hussain (1990) found that plaque size was directly correlated to the pathogenic effect of LI viruses in mice. Furthermore, a similar association was reported with DEN and JE virus (Eckels *et al.*, 1976; Harrison *et al.*, 1977; Sumiyoshi *et al.*, 1995) as well as other virus species, e.g. swine vesicular disease virus (Niemaltowski, 1983). However, the previous investigation of the biological characteristics of LI viruses only involved three viruses, while this study has identified a more complex situation by analyzing ten LI viruses.

5.3.3. Effect of Mouse Strain

When LI viruses were compared in C57/BL and SW mice, the onset of disease was 1-3 days earlier in SW mice and a greater number of fatalities occurred. Furthermore, the neurological symptoms observed in SW mice prior to death or euthanization were more pronounced than those in C57/BL mice. These observations imply that LI virus replicates more rapidly in SW mice and that C57/BL mice may be partially resistant to LI virus infection. Similar results have been reported for YF virus strains which exhibited different virulence characteristics in various mouse strains (Liprandi, 1981; Ryman *et al.*, 1997).

A single autosomal dominant gene (*Flv*^{r}) located on chromosome 5 of the murine genome was found to convey resistance against flavivirus infection in wild mice (*Mus musculus*) and several laboratory strains (Sabin, 1952; Darnell *et al.*, 1974; Green, 1989; Sangster *et al.*, 1994). The resulting gene product was not involved in the immune response (Darnell *et al.*, 1974; Brinton *et al.*, 1982) and did not affect the ability of cells to be infected (Darnell & Koprowski, 1974). However, after 24 hours the virus yield was lower in cultured cells from resistant mice than in cells from susceptible mice, implying that the *Flv* gene product affects the multiplication of flaviviruses (Darnell & Koprowski, 1974). It was proposed that a higher proportion of defective particles, containing truncated RNA, was produced in resistant cells. These particles interfere with the multiplication of normal virus resulting in lower yields, providing the host immune response time to act (Smith, 1981; Brinton, 1983). However, in a recent study of MVE virus infection of susceptible and resistant mice, truncated RNA was not identified, although RF and RI forms of RNA accumulated to a greater extent in resistant mice (Urosevic *et al.*, 1997). This suggested that the *Flv* gene product interferes with flavivirus replication in an unknown manner.

However, C57/BL mice do not encode the dominant *Flv* gene. A study investigating the virulence characteristics of JE virus in C57/BL and C3H/He mice suggested that the former were more resistant to infection and this resistance was under the control of a single dominant gene separate from *Flv* (Miura *et al.*, 1990). This gene has not been implicated in the resistance of C57/BL mice to any other

flavivirus. However, the lower level of susceptibility to LI virus detected in C57/BL mice in this study implies that the involvement of this gene cannot be discounted.

5.3.4. Effect of Mouse Gender

In addition to the mouse strain influencing the pathogenicity results it appears that the gender of the mouse may also influence the outcome of infection. Although the experiment involved relatively small numbers of animals, INV6 and PRES1 virus caused higher mortality in female than male animals, while PEN3 virus caused higher mortality in male than female C57/BL mice. It is interesting to note that although INV6 virus caused considerably different mortality in female and male C57/BL mice a similar result was not observed in SW mice. Female SW mice developed symptoms before males but an equal number of mice of each gender died.

It is possible that the variation in susceptibility of LI virus observed in female and male C57/BL mice could be due to the size of the mouse. Male C57/BL mice are larger than females. However, this cannot explain the mortality observed with PEN3 virus where males were more susceptible than females. A DEN virus NS1 protein vaccine induced greater protection in female mice than male animals (Qu *et al.*, 1993) and it was proposed that the greater immune reactivity of female mice was responsible for this difference. The involvement of genes, possibly situated on the sex chromosomes, may be responsible for the variation detected in male and female C57/BL mice. However, without extending these data to include a more statistically significant number of animals the importance of the gender difference results described in this study cannot be elucidated.

5.3.5. Effect of Mouse Age

Susceptibility of mice to flaviviruses is also age dependent (O'Leary *et al.*, 1942; Sabin, 1952; Smith, 1981). Intraperitoneal inoculation of hamsters with LI virus

results in disease in animals less than 10 days old but after this age the infection is asymptomatic (Doherty, 1969a). This variation may be due to the immune status of the animal. Alternatively, the maturity of neurones may be of importance. Fischer rats are resistant to JE virus infection when 13 days of age. However, if intracerebral neurones from embryos are transplanted into 15 day old rats, before inoculation with JE virus, then viral antigens are detected in the transplanted neurones (Ogata *et al.*, 1991).

The age of the C57/BL mice included in this study may have influenced the outcome of the experiments. The virus titres were measured in mice aged 5-7 weeks while the mortality experiment involved 3-5 week old C57/BL mice. The older mice may have been more resistant to LI virus infection, which therefore resulted in the detection of lower virus titres in the brains of mice compared with those observed in a previous study (Doherty, 1969b). The resistance of C57/BL mice for JE virus infection described by Miura *et al.* (1990) was observed in 10-17 week old mice. This resistance may only become apparent once the mice reach maturity and therefore is only observed in mice over five weeks of age. However, LI viruses caused disease earlier in 3-5 week old SW mice than C57/BL mice of the same age and resulted in higher mortalities. These results imply that C57/BL mice are partially resistant to LI virus infection. Unfortunately, time constraints prevented additional studies of the virus titres in infected 3-5 week old C57/BL mice.

5.3.6. Genetic Determinants of Virulence

There were no unique substitutions shared within the E proteins of IRE2, IRE3 and INV1 virus, the three most pathogenic LI viruses investigated. In addition there was no shared unique sequence between the two most attenuated viruses, PEN6 and LI/A. However, a correlation between geographic distribution and virulence characteristics was identified. Viruses from Ireland, representing both Irish and British LI viruses, were among the most pathogenic, both *in vivo* and *in vitro*. An association between antigenic type and pathogenicity was discussed in chapter 3. In summary,

substitutions at residues 308, 311, 346 and 356 of the E protein, which are associated with the antigenic characteristics of the viruses, were implicated as virulence determinants. The substitution of Asp-308 to Glu resulted in virus attenuation, while additional substitutions at residues 311, 346 or 356 appeared to neutralize the effect of Glu-308 to varying degrees and resulted in more virulent viruses.

The importance of the alternative species-specific marker was also investigated. Variation in the species-specific marker was previously observed among TBE viruses. WTBE and FETBE viruses encode the genetic marker AQN, while the attenuated Vs strain of TBE virus encodes the motif AQQ (Gritsun *et al.*, 1993a). The importance, if any, of the Gln-234 residue in the attenuation of Vs virus is unclear. However, it is interesting to note that an unattenuated escape variant of WTBE virus selected in the presence of MAb A4 encoded an altered species-specific marker AKN (Mandl *et al.*, 1989a; Holzmann *et al.*, 1990). Therefore substitutions at residue 233 affect the structure of the protein and it is possible that substitutions at residue 234 may have a similar effect. The Asn-234 to Gln substitution of Vs virus is conserved and therefore significant structural effects may not occur. However, the substitution of His-234 to Tyr observed in several LI viruses is not physicochemically conserved.

PEN3 and PEN6 virus were isolated from the same farm in the same year and differ at only two amino acid residues within the E protein, including the His or Tyr residue of the species-specific marker. These two viruses differed considerably in virulence for C57/BL mice. The NPH containing PEN3 virus resulted in mortality in 70% of mice while PEN6 virus, with the genetic marker NPY, caused 25% mortality. However, the type-I escape variant INV6, which is also an NPY containing virus, caused similar mortality levels as PRES1 virus, the other type-I escape variant investigated, implying that residue 234 is not a virulence determinant. PEN6 virus differs from PEN3 virus at only one additional residue within the E protein, the substitution of Thr-471 to Ser, which is also encoded in the pathogenic Irish LI virus, MA54. Therefore the genetic determinants of attenuation encoded in PEN6 virus probably lie outside the investigated E gene.

This investigation of the biological characteristics of LI virus has illustrated extreme diversity among the viruses. However, there was little detectable association between the sequence of the E protein and the virulence characteristics of the viruses, with the possible exception of residues involved in the antigenic properties of the viruses. These results imply that LI virus virulence is multigenetic and the investigation of other regions of the genome is vital to understand the virulence determinants of flaviviruses.

Virus	*In vitro*	*In vivo*		Characteristic of interest
		C57/BL	SW	
DEV1	+	-	-	type-II escape variant
DEV2	+	-	-	type-II escape variant
DEV3	+	-	-	type-II escape variant
DEV4	+	+	-	type-II escape variant
INV1	+	+	-	type-II escape variant
INV6	+	+	+	type-I escape variant/NPY
INV14	+	-	-	
IRE2	+	+	+	Irish LI virus
IRE3	+	+	-	British LI virus
LI/369	+	-	-	
LI/A	+	+	-	type-II escape variant
LOCH2	+	-	-	
LOCH6	+	-	-	
PEN3	+	+	-	NPH
PEN4	+	-	-	NPY
PEN6	+	+	+	NPY
PRES1	+	+	-	type-I escape variant
SB/526	+	+	-	
THO2	+	-	-	

Table 5.1. LI viruses chosen for further investigation by; *in vitro* analysis involving plaque morphology, and *in vivo* studies in C57/BL and SW mice. + denotes that the analysis in question was carried out, - denotes that the analysis was not carried out using that particular LI virus.

Virus	Mean Area ± Standard Error (mm^2)	Range (mm^2)	Antigenic Type	Country of Origin
IRE2	5.98 ± 1.20	4.13-8.38	wild-type	Ireland
IRE3	5.58 ± 1.64	2.88-7.69	wild-type	Ireland
LOCH2	5.39 ± 1.87	2.69-8.63	wild-type	Scotland
THO2	4.51 ± 0.85	3.31-6.19	wild-type	Scotland
SB/526	4.19 ± 1.10	2.75-6.38	wild-type	Scotland
LI/369	3.70 ± 0.79	2.25-4.81	wild-type	Scotland
LI/A	3.33 ± 1.23	1.13-5.25	type-II	England
LOCH6	3.15 ± 0.81	2.00-4.56	wild-type	Scotland
INV6	3.12 ± 0.35	2.63-3.75	type-I	Scotland
PEN3	3.05 ± 0.84	2.06-5.19	wild-type	England
DEV4	2.97 ± 0.45	2.25-3.94	type-II	England
INV14	2.94 ± 0.67	1.44-3.88	wild-type	Scotland
DEV3	2.88 ± 0.57	2.06-3.94	type-II	England
PRES1	2.87 ± 0.28	2.50-3.31	type-I	England
DEV2	2.77 ± 0.47	1.81-3.50	type-II	England
PEN4	2.71 ± 0.81	1.06-4.25	wild-type	England
PEN6	2.36 ± 0.71	1.13-3.63	wild-type	England
INV1	2.03 ± 0.48	1.00-2.50	type-II	Scotland
DEV1	1.98 ± 0.36	0.94-2.25	type-II	England

Table 5.2. LI viruses listed in descending order of mean plaque size (mm^2) produced on PK cell monolayers after 96 hours incubation. The table includes information on the general location where the viruses were collected and the antigenic phenotype as assigned according to the IIF analysis (Table 3.1).

Virus	Day 1	Day 3	Day 5	Day 7
DEV4	0	0.32 ± 0.64	0	0
INV1	0.59 ± 0.72	0.89 ± 0.73	0	0
INV6	0.42 ± 0.54	0.30 ± 0.61	0	0
IRE2	1.38 ± 0.73	1.49 ± 0.51	0	0
IRE3	1.80 ± 0.27	0.88 ± 0.80	0.74 ± 0.75	0
LI/A	0.59 ± 0.72	1.00 ± 0.56	0	0
PEN3	0.65 ± 0.55	0	0	0
PEN6	0.83 ± 0.70	0	0	0
PRES1	1.01 ± 0.60	0.16 ± 0.33	0.50 ± 0.67	0
SB/526	0.39 ± 0.49	0.16 ± 0.33	0	0

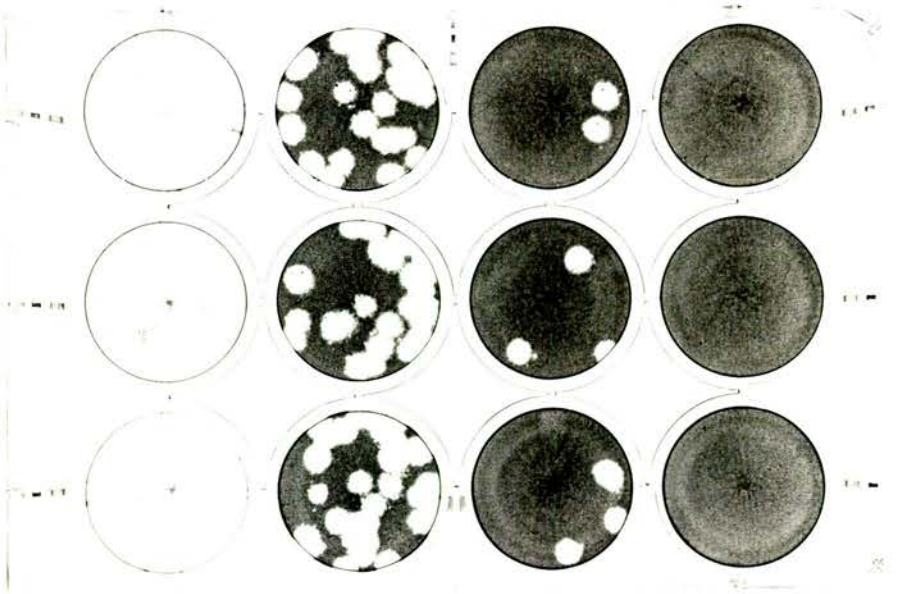
Table 5.3 Mean ± standard error viraemia titres (log_{10} p.f.u./0.2 ml) detected in C57/BL mice during the first 7 days post inoculation with LI viruses.

	C57/BL Mice				SW Mice
Virus	Day 1	Day 3	Day5	Day 7	Day 6
DEV4	0	0	0	0	*
INV1	0	0	0	0	*
INV6	0	0	0	0	5.89 ± 1.68
IRE2	0	0	0.36 ± 0.73	0.81 ± 0.65	7.25 ± 1.60
IRE3	0	0	0.36 ± 0.73	1.33 ± 1.63	*
LI/A	0	0	0	0	*
PEN3	0	0	0	0	*
PEN6	0	0	0	0	4.31 ± 2.21
PRES1	0	0	0.52 ± 1.04	0.68 ± 1.35	*
SB/526	0	0	0	0	*

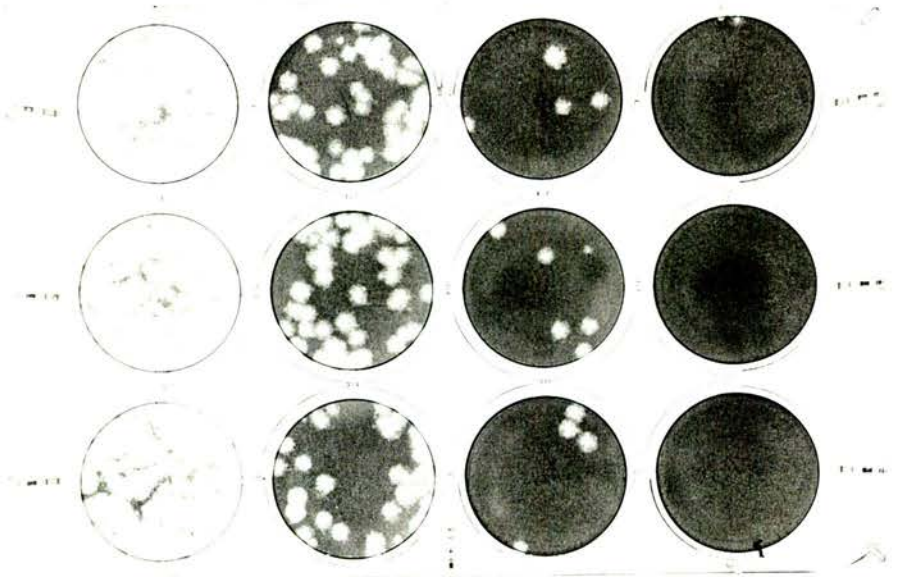
Table 5.4. Mean ± standard error titres of virus (log10 p.f.u./0.2 gm) detected in the brains of C57/BL and SW mice. * indicates that no virus isolation was attempted.

Fig. 5.1. Variation in the morphology of plaques formed by selected LI viruses on PK cell monolayers 96 hours post inoculation (actual size). a) IRE2 virus, b) PEN3 virus and c) INV1 virus.

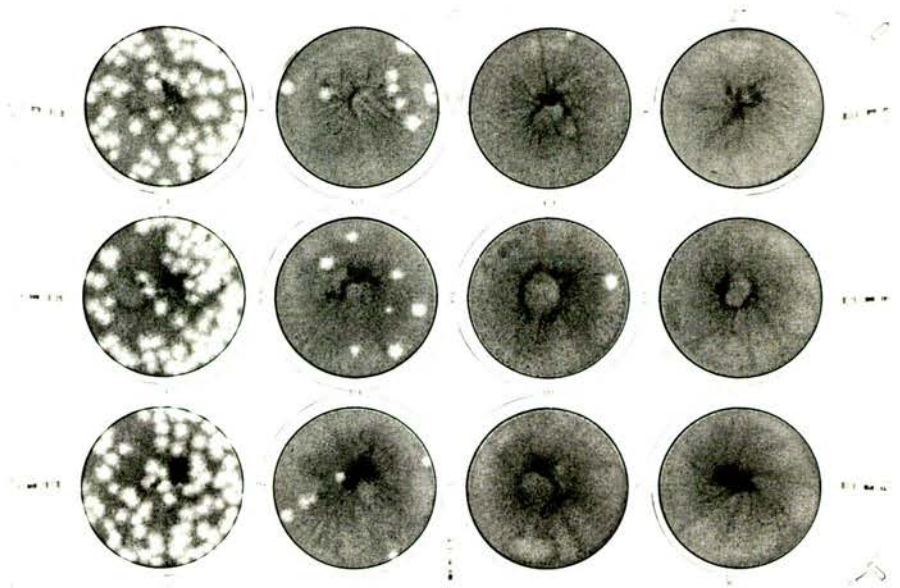
a)



b)



c)



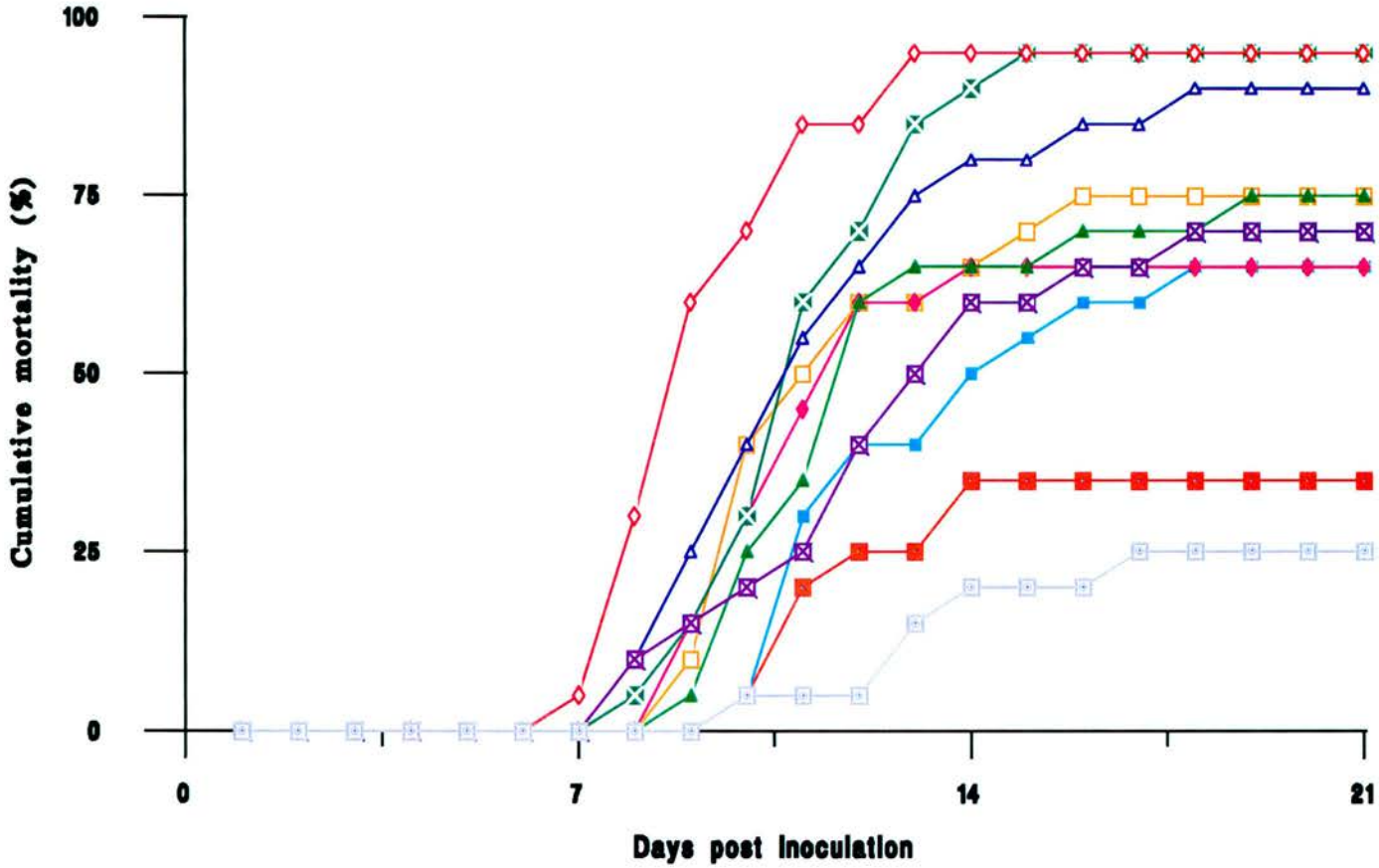


Fig. 5.2. Cumulative mortality of three week old C57/BL mice inoculated with LI viruses; DEV4 (—■—), INV1 (—✕—), INV6 (—◆—), IRE2 (—◇—), IRE3 (—△—), LI/A (—■—), PEN3 (—⊠—), PEN6 (—□—), PRES1 (—▲—) and SB/526 (—□—).

Fig. 5.3a. DEV4 virus

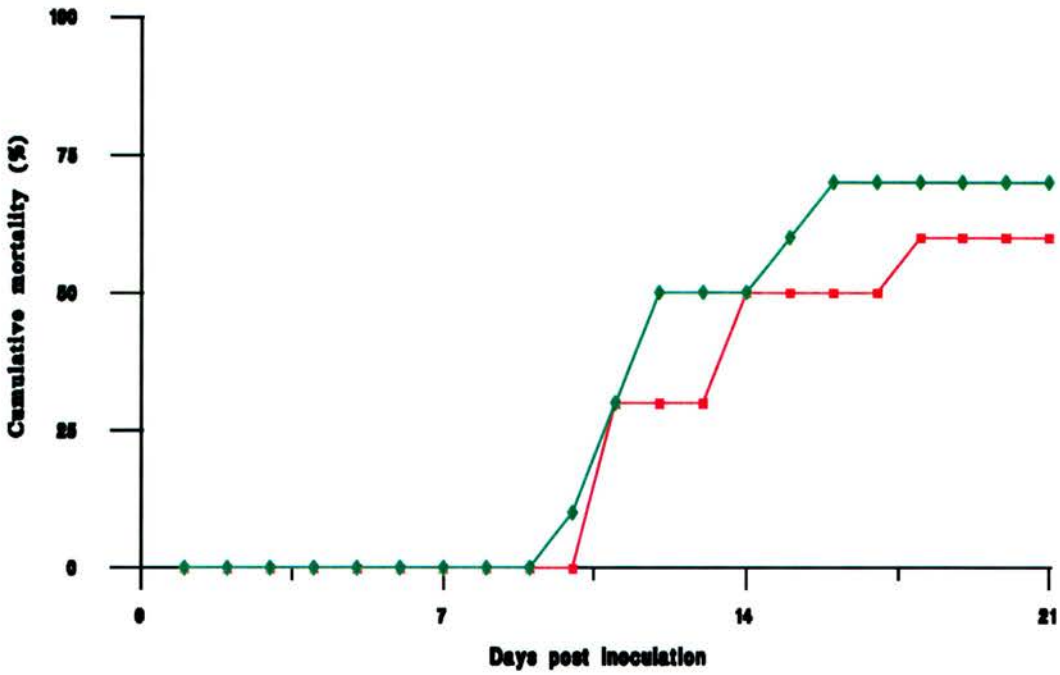


Fig. 5.3b. INV1 virus

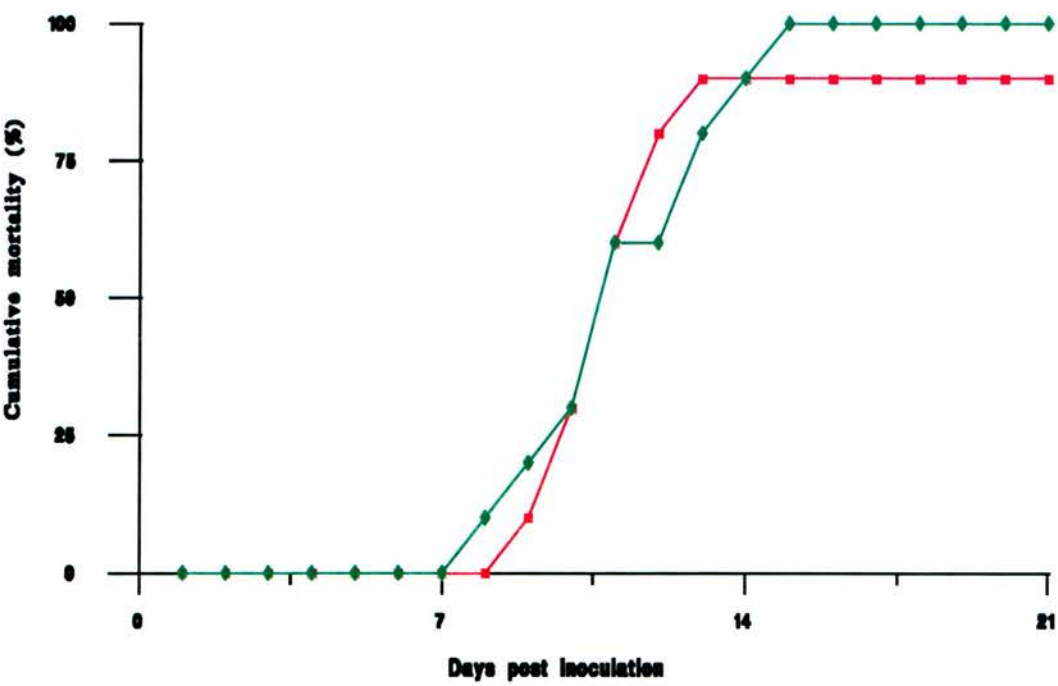


Fig. 5.3. Cumulative mortality of 3 week old male (—■—) and female (—◆—) C57/BL mice inoculated with LI viruses a) DEV4, b) INV1, c) INV6, d) IRE2, e) IRE3, f) LI/A, g) PEN3, h) PEN6, i) PRES1 and j) SB/526.

Fig. 5.3c. INV6 virus

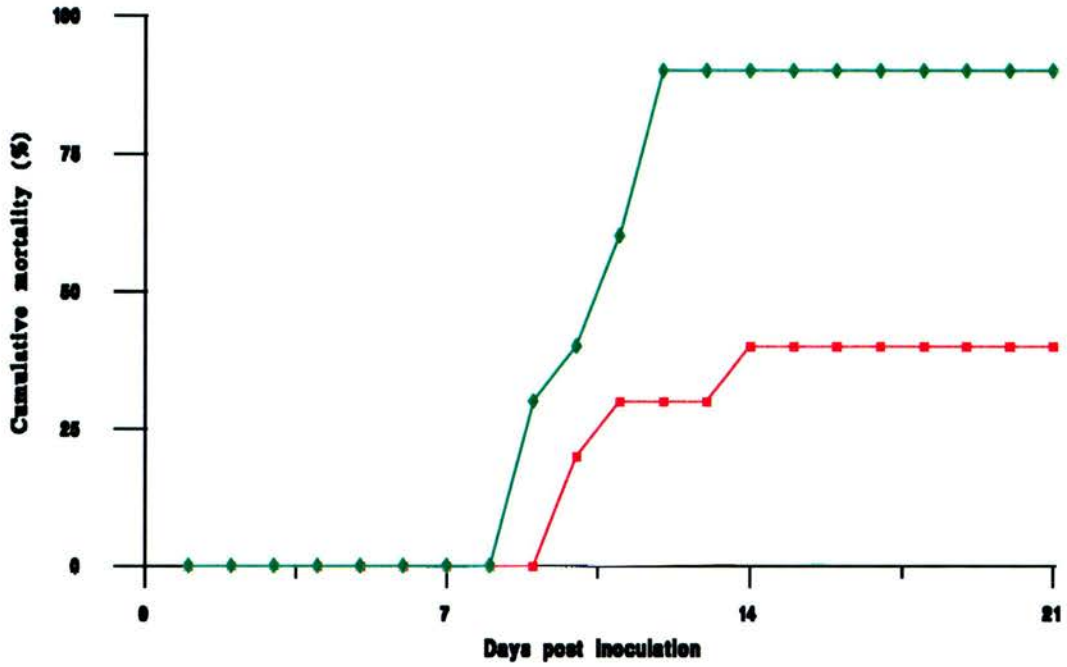


Fig. 5.3d. IRE2 virus

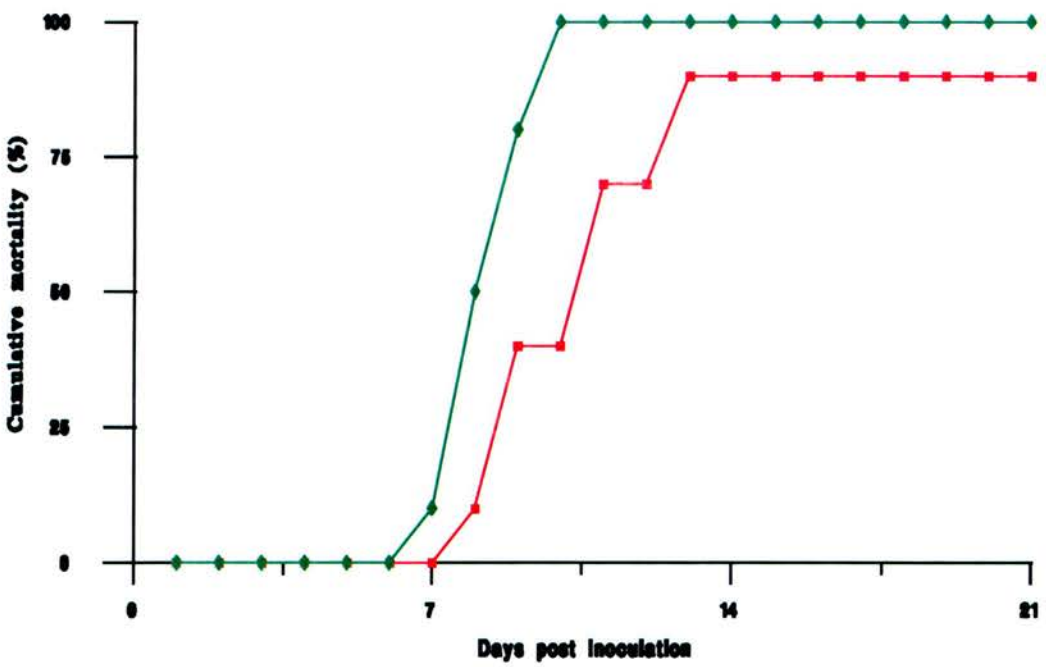


Fig. 5.3e. IRE3 virus

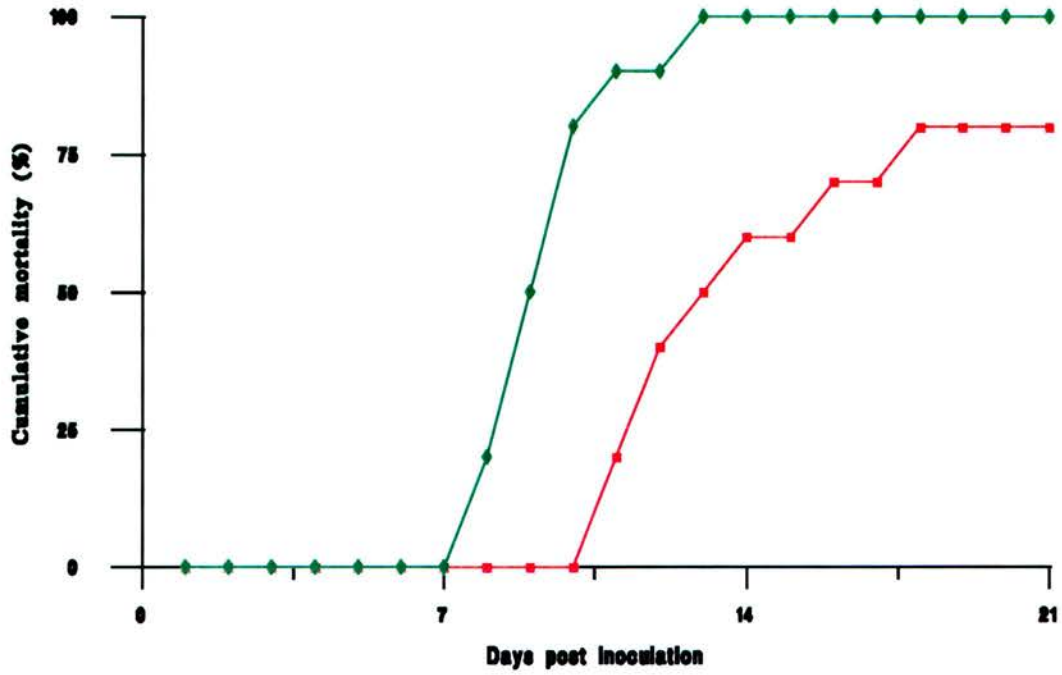


Fig. 5.3f. LI/A virus

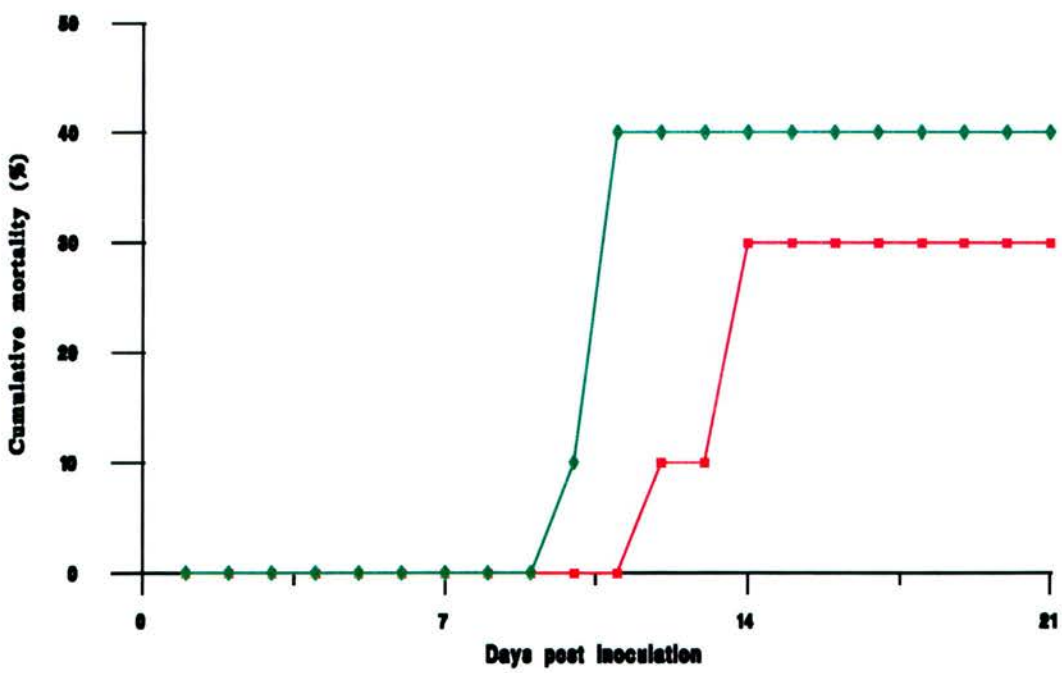


Fig. 5.3g. PEN3 virus

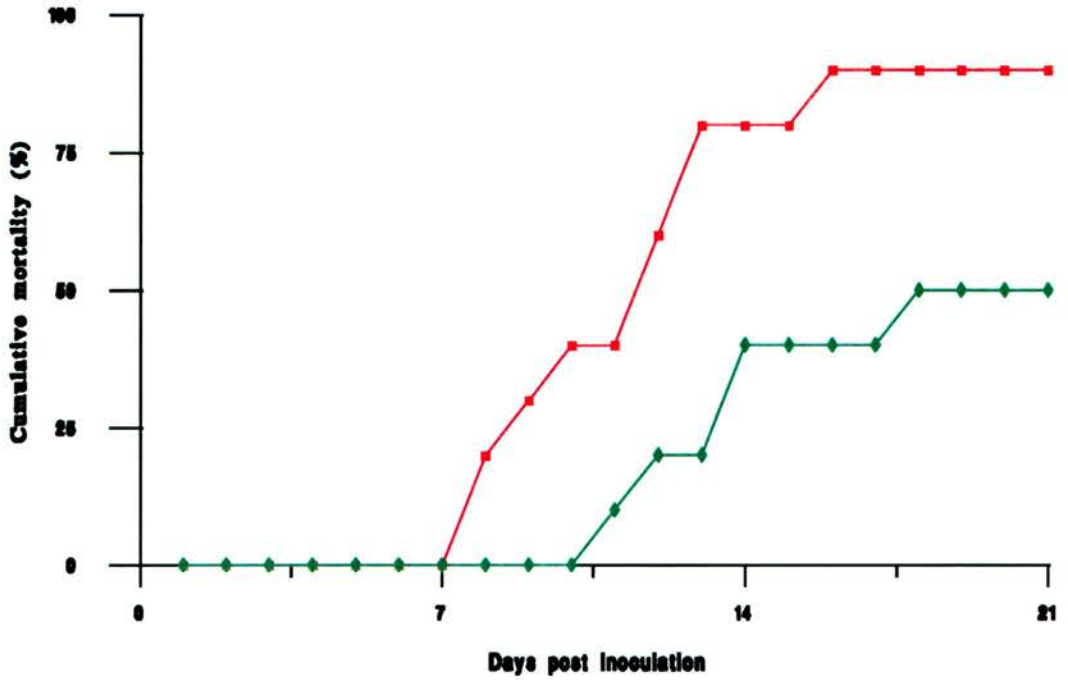


Fig. 5.3h. PEN6 virus

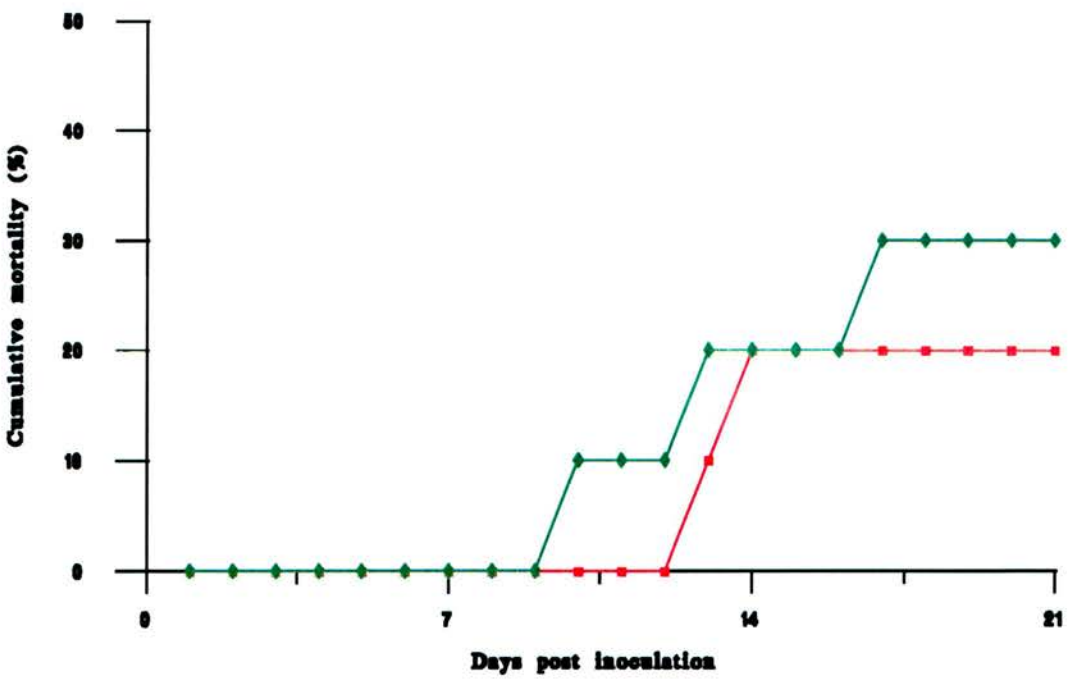


Fig. 5.3i. PRES1 virus

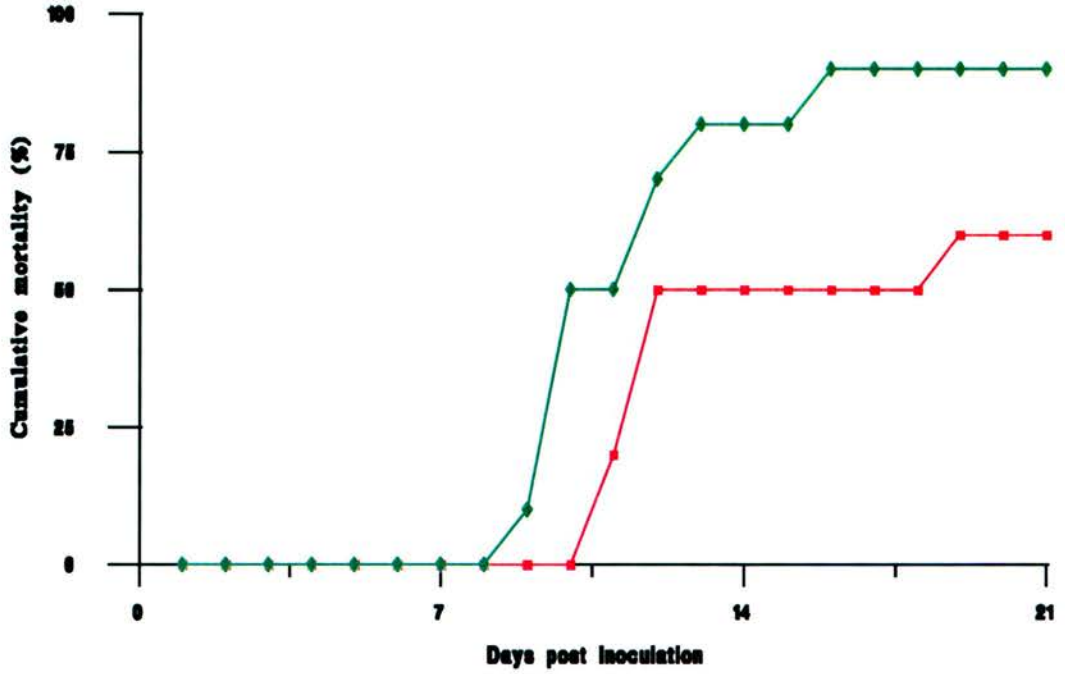
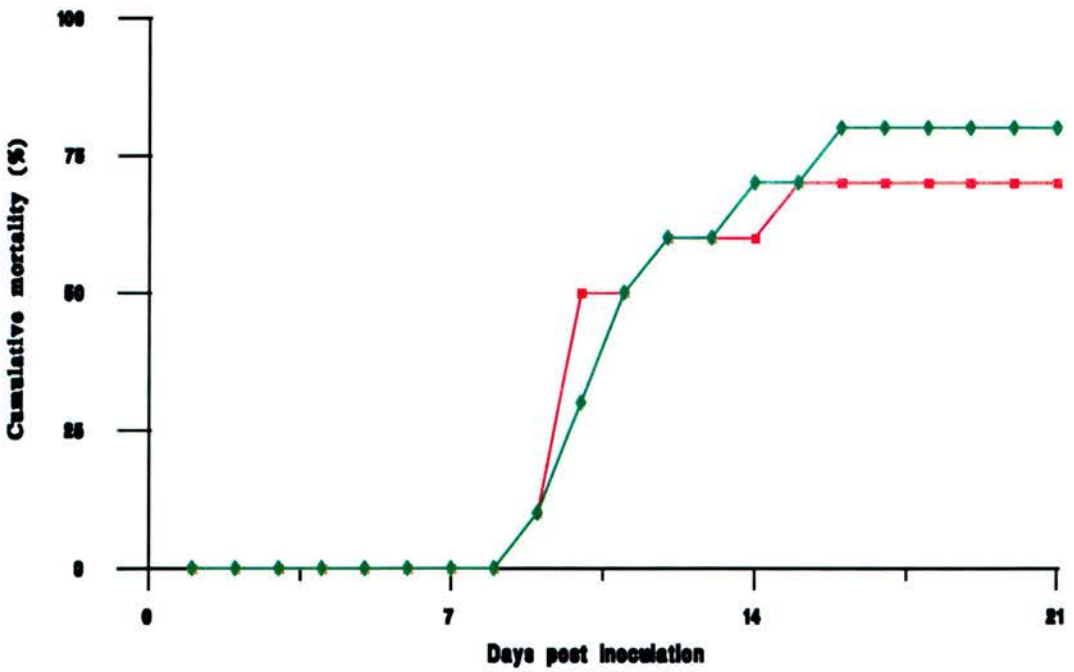


Fig. 5.3j. SB/526 virus



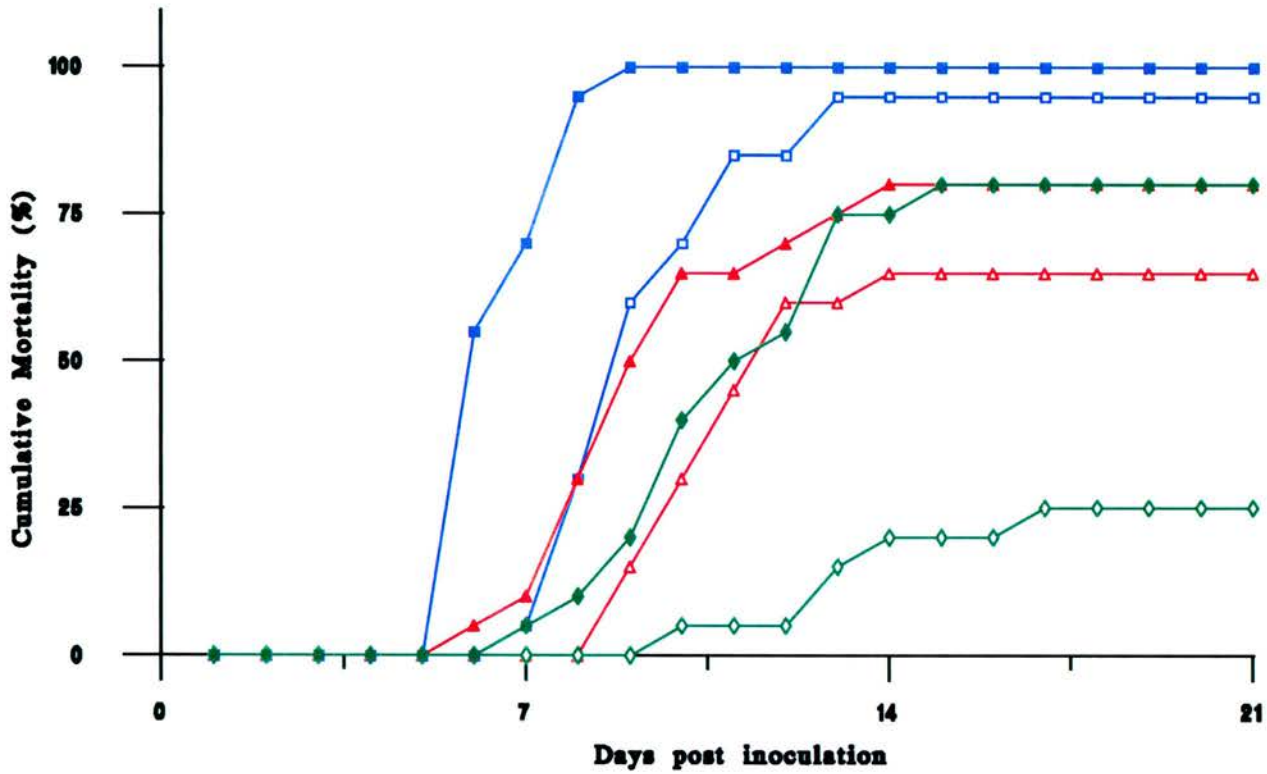
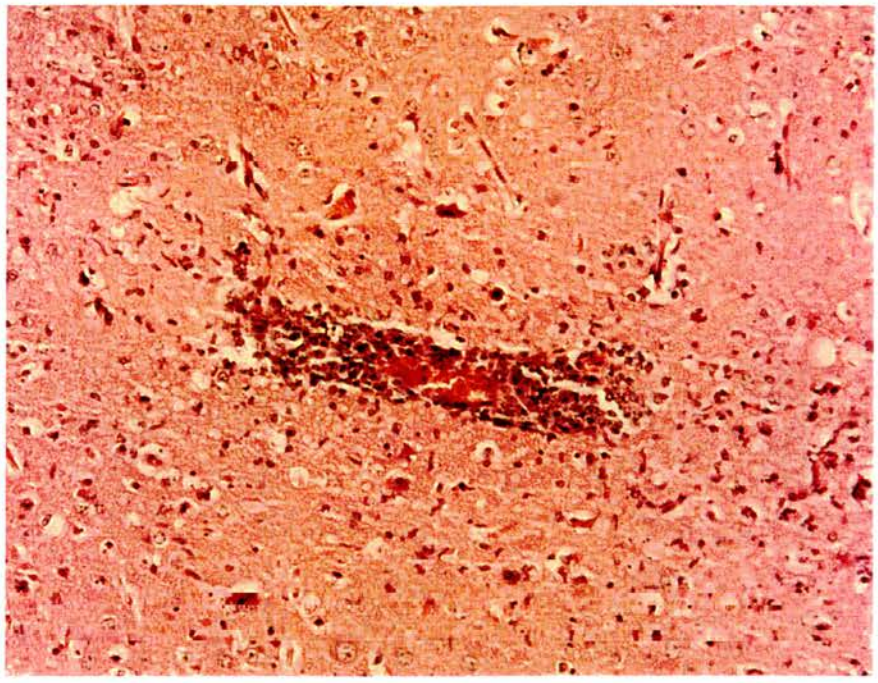


Fig. 5.4. Comparison of the cumulative mortality of three week old C57/BL and SW mice inoculated with INV6 virus (—△— for C57/BL & —▲— for SW mice), IRE2 virus (—□— for C57/BL & —■— for SW mice) and PEN6 virus (—◇— for C57/BL & —◆— for SW mice).

a)



b)

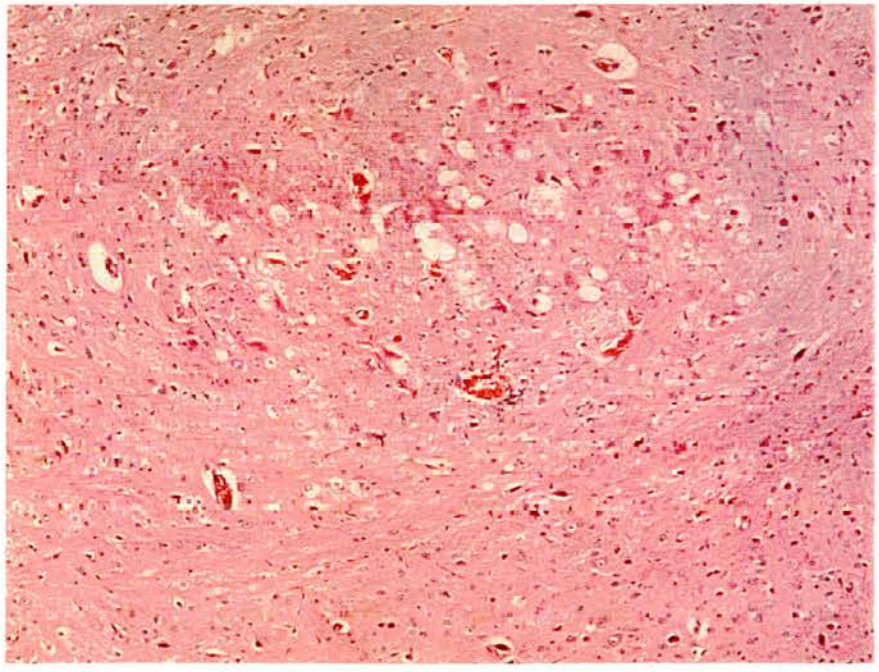


Fig. 5.5. Histopathological changes observed in SW mice 12 days after subcutaneous inoculation of the LI virus PEN6; a) perivascular cuffing observed in the forebrain (x20 magnification, H&E), b) spongiform change observed in the midbrain (x10 magnification, H&E)

CHAPTER SIX

General Discussion

CONTENTS

6.1. Discussion

Because of its unique position as the only flavivirus in the British Isles and also because the virus rarely causes disease in humans, LI virus has been studied mostly by British and Irish veterinary virologists and has largely been ignored by other virologists world-wide. However, the virus is of interest because of its economic importance in moorland areas where sheep farming and game bird management represent a large proportion of the local income, and because of its evolutionary importance as the youngest flavivirus recognized to date. Furthermore, a large collection of LI viruses have been collected over the last 60 years which represent the virus population found throughout the British Isles. This collection of viruses therefore provided the opportunity for a detailed study of LI virus pathogenic, antigenic and genetic variation as well as its epizootiological and evolutionary characteristics.

LI virus differs from most of the other viruses in the TBE virus serocomplex in that it is not associated with the forest ecosystem. LI virus occurs on moorland and like SSE and TSE virus it has evolved a life-cycle which is almost exclusively dependent upon the sheep and the tick, as opposed to rodents and ticks with which the forest viruses are normally associated. A recent phylogenetic analysis implied that LI virus evolved from WTBE virus only 300 years ago (Zanotto *et al.*, 1996b). This hypothesis questions the traditional belief that LI virus or the ancestral virus has existed in the forests of the British Isles since the last ice-age and raises the additional question, how did LI virus reach the British Isles from Europe and become established on the moorland habitat?

It is surprising that LI virus has not genetically reverted back to become a virus which utilizes the forest ecosystem, a niche which appears to be unoccupied in the British Isles. This may simply be a matter of time. On the other hand, the British forest ecosystem may not provide sufficient genetically susceptible vertebrate species to sustain the virus. Nevertheless, considering the widespread livestock movements, which have been implicated in the dispersal of LI virus, there appear to have been

plenty of opportunities for the virus to be introduced into woodlands or at least the forest fringes.

Another interesting feature of these viruses is that although LI and other sheep encephalomyelitic flaviviruses are genetically distinct, they are antigenically virtually indistinguishable. Therefore the sheep-tick cycle must select for particular genetic features and have a constraining effect on the evolution of the virus proteins once the virus of highest fitness has been selected.

One of the most intriguing features of LI virus biology is the lack of human disease. LI virus can cause severe disease and even fatalities in humans but there have only been 45 reported cases of disease and more than half were the result of laboratory infections (Davidson *et al.*, 1991). This is surprising considering the number of people who work on moorland and the popularity of hill-walking and outdoor pursuits. Therefore a factor or factors must be involved in limiting the disease. Possibly LI virus is attenuated in humans and therefore usually only results in mild flu-like symptoms which go undetected. One of the most likely explanations is the low probability of humans becoming infected. LI virus is present in only a low proportion of ticks, less than 1 in 600 (Varma & Smith, 1971) and the tick burden of any human is likely to be very low. In addition, ticks will usually be quickly removed once detected and therefore insufficient time may have elapsed for the virus to be transmitted with the tick saliva.

Previous studies investigating the variation amongst LI viruses have raised a number of important and interesting questions about the virus. This study has attempted, and largely succeeded, in providing definitive answers for a number of these questions which were outlined in chapter one (section 1.9).

6.1.1. Antigenic Analysis of LI Viruses.

The antigenic homogeneity of the LI viruses was demonstrated by IIF analysis using a panel of fourteen E and NS1 protein-specific MAbs. MAbs can detect differences between the viruses that represent single amino acid substitutions. However, British

and Irish LI viruses, which share only 95.4-96.4% amino acid sequence similarity in the complete E protein, were not distinguished by antigenic analysis.

The two host life-cycle of the virus is probably largely responsible for the lack of antigenic variability observed amongst the LI viruses for several reasons. Firstly, LI virus replication in vertebrate hosts is a transient phenomenon lasting, in the case of sheep or grouse, only a few days. Other vertebrate species may or may not be infected for longer periods of time but the titres attained in these vertebrates are usually relatively low (Reid, 1988). Thus overall, virus turnover in vertebrate hosts, during the life-cycle, is relatively low. The implication of this is that there is only a limited opportunity for the selection of variants under immune selective pressure. Secondly, LI virus spends most of its time in ticks and appears to replicate only to a limited extent over periods of up to one year or more. During this extended time, the virus must survive significant changes in the physiology of the tick and successfully infect cells in the salivary gland tissue to ensure its transfer to a vertebrate host. Since there is no conventional immune system in the tick, the constraints on the virus will be largely confined to those affecting the conformation of the structural proteins (influencing virus tropism and pathogenesis) and the specific requirements of the tick for enzymatic activity by the nonstructural virus proteins. It seems reasonable to assume that any changes that occur during the brief period of existence of the virus in the vertebrate host will be selected against when the virus is introduced into another tick. Most substitutions which persist in the virus population are physicochemically conserved (Zanotto *et al.*, 1995). Thirdly, there is now convincing evidence that LI virus can be transmitted from a donor to a recipient tick through the mechanism of tick co-feeding (Jones *et al.*, 1997). Under these circumstances, the vertebrate host may effectively act as the carrier of the virus for only very limited periods of time. When co-feeding occurs, there may be no detectable viraemia and therefore virtually no opportunity for variation to occur in the vertebrate host.

Despite these arguments antigenic variation, in the form of altered binding properties of LI viruses, was observed in two E protein-specific MAbs, 4.2 and 7.1. These were the only neutralizing MAbs obtained against LI virus (Hussain, 1990), despite repeated attempts to prepare additional neutralizing MAbs (Gould, pers.

com.). The amino acid substitutions responsible for the altered antigenicity lie within a variable region of the E protein (Gritsun & Gould, 1995) which is located on the outer surface of the virus particle (Rey *et al.*, 1995). At such sites structural changes can probably occur without modifying the overall structure of the protein and therefore do not affect the fitness of the virus during replication in the tick.

The antigenic homogeneity of LI viruses implies that the commercially available inactivated LI virus vaccine, which is produced using LI/31 virus, should induce protective immunity against all LI viruses found in the British Isles. Nevertheless, the virus persists in the environment. The first LI virus vaccine produced by the Moredun Research Institute failed to stimulate detectable levels of antibody against LI virus, but sensitized the animal to infection (Williams & Thorburn, 1961; Smith *et al.*, 1964a). As a result an immune response was quickly generated after natural infection, thus protecting the animal from disease. However, viraemic titres still reached the threshold levels required for uptake of virus by ticks and therefore the virus could persist within the sheep-tick cycle (Williams & Thorburn, 1961; Smith *et al.*, 1964a). The LI virus vaccine presently in use induces high levels of HI and neutralizing antibodies after two doses (Shaw & Reid, 1981). However, as noted above, experimental transmission of LI virus can occur between ticks co-feeding on mountain hares, even on animals which had seroconverted (Jones *et al.*, 1997). Mountain hares can harbour large numbers of ticks although it is estimated that 80-90% ticks feed on sheep in moorland areas (Milne, 1949). Therefore, if co-feeding occurs on seroconverted sheep, even at low levels, the persistence of the virus in many areas could be explained on the basis of co-feeding.

Two types of naturally occurring escape variants were observed amongst the LI viruses and the amino acid substitutions responsible for these changes were identified. The amino acid substitutions involved are examples of Darwinian positive selection (Zanotto *et al.*, 1995). The altered phenotypes may improve the fitness of the virus for vertebrate hosts and have been independently selected in several viral lineages. The escape variant phenotype appears to be fixed in the virus population, as illustrated by the Devon viruses, implying that the altered structure does not reduce the fitness of the virus during replication in the tick. Although

similar escape variants were selected experimentally in the laboratory using MAb 4.2, the vertebrate host immune system may not be the selection pressure involved under natural conditions. The responsible substitutions, at residues 308 and 311, lie within domain III of the E protein and form part of the IgC-like putative receptor binding domain (Rey *et al.*, 1995). Therefore the resulting conformational change in the escape variants may alter the receptor specificity, i.e. the tropism, of the virus and only incidentally affect the phenotype detected by MAbs 4.2 and 7.1.

Whatever selection pressures are involved in the generation of escape variants, these must vary between different geographic locations. Escape variants have not been isolated in Ireland nor from the west of Scotland, while all viruses from Devon are type-II escape variants. It is interesting to note that the Scottish virus INV1, which is most closely related to the Devon viruses, is also a type-II escape variant. Since all Devon viruses are closely related, a single successful introduction of the virus, as a quasispecies, is presumed to have occurred. These observations imply that the initial virus introduced into the south-west of England was a type-II escape variant from the east of Scotland. If this hypothesis is correct, naturally occurring escape variants existed during the first half of the century, when the Scottish and Devon virus lineages are estimated to have diverged, although escape variants have only been isolated since 1980. However, the lack of earlier escape variants may simply be due to sampling bias and the low number of viruses collected before 1980. The fact that the generation of escape variants may have occurred in the first half of the 20th century implies that the use of vaccines has not been the major selection pressure involved in escape variant generation because the vaccine was only developed in the 1930s, at the same time as the first reported cases of LI disease in Devon (Reid, pers. com.).

6.1.2. Genetic Determinants of LI Virus Virulence

Although relatively low levels of antigenic and genetic variation were detected between the viruses studied, they showed profound differences in their biological

characteristics, both *in vivo* and *in vitro*, with the virulence of these viruses for C57/BL mice varying from low (25% mortality) to high (95% mortality). Irish LI viruses were consistently among the most pathogenic, in this and previous studies (Hussain, 1990; Gao *et al.,* 1994). Moreover, the British LI virus that was identified in Ireland was also highly virulent for mice. Evidence of an association between the virulence characteristics of the viruses and their genotypes or antigenic phenotypes was looked for but only very tenuous associations were identified. For example, three type-II escape variants; LI/A, DEV4 and INV1, and the type-I escape variant INV6, were shown to be genetically very closely related but differed considerably in mouse virulence and this may be associated with single amino acid substitutions at residues 311, 346 and 356. However, without determining the full genome sequence of these LI viruses the involvement of such residues in virulence characteristics cannot be verified. LI/A and DEV4 virus share 99.7% nucleotide sequence similarity in the E gene, however, a single substitution in any other gene could result in attenuation. For example, the nucleotide substitutions responsible for the attenuation of a DEN type-2 virus vaccine candidate have been mapped to the prM, NS1, NS2a and NS4a genes and the 5'-NCR (Kinney *et al.*, 1997). In addition, a single amino acid substitution in the NS5 protein of YF virus resulted in altered neurovirulence characteristics in mice (Xie *et al.*, 1996). These and many other similar observations indicate that whilst virulence, under a very defined set of conditions, may be determined by single nucleotide substitutions, it is a multigenetic phenomenon, i.e. the same level of attenuation for a particular virus may be obtained by different substitutions, either single or multiple.

Comparisons of virulence for mice with the different LI viruses were interesting for several reasons. The mouse strain and gender affected the outcome of the mortality experiments. The observation that female C57/BL mice were more susceptible to infection by LI viruses than male C57/BL mice was both surprising and difficult to explain. This was made even more difficult because in one case male mice were more sensitive to LI virus infection than female mice. Whilst relatively large groups of mice were used in the experiments, it seems prudent at this stage to conclude that the experiments should be repeated more times and under different

conditions before attempting to draw conclusions. SW mice were more sensitive to LI virus infection than C57/BL mice. LI viruses which showed high virulence in SW mice were also virulent in C57/BL mice. Therefore, these results imply a genetic basis for the partial resistance of C57/BL mice to infection by LI virus, as previously suggested for JE virus (Miura *et al.,* 1990).

6.1.3. Investigation of the Potential Usage of Genetic Markers

Three genetic markers were previously identified in the E gene (Shiu *et al.,* 1991, 1992a; Gao *et al.,* 1993a). No variation in the two TBE serocomplex specific markers, EHLPTA and DSGHD, was detected in the LI viruses included in this study. However, three forms of the tripeptide species-specific marker; GPR, NPH and NPY, were identified.

The tripeptide marker can be used to distinguish between flavivirus species but failed to distinguish between phylogenetically distinct populations of LI virus, e.g. between British and Welsh LI viruses. If the tripeptide is extended to a pentapeptide, four forms of the species-specific marker are identified which are in general agreement with the phylogenetic analysis. However, the presence of His or Tyr at position 234 of the pentapeptide is not associated with phylogeny. Use of the pentapeptide for classification purposes would result in viruses with the genetic marker DGNPY being grouped together, although the viruses represent separate viral lineages from Scotland and the north of England. This again highlights the problem of phylogenetic analysis of closely related viruses by studying the amino acid sequence. Therefore, although the genetic marker can be used to categorize the flaviviruses down to the sub-species level, phylogenetic analysis of longer regions of E gene nucleotide sequence, e.g. the RGF, provides more suitable data for investigating the classification of the flaviviruses as they reveal more evolutionary information.

Nevertheless, genetic markers of the type described could prove invaluable in diagnostic laboratories with only limited molecular resources. Once the E gene

sequences have been determined for all flaviviruses, sequencing a small region of the viral genome would immediately and absolutely identify any of the flaviviruses within the genus. Moreover, at this stage there is no recognized biological function associated with the genetic markers. It seems reasonable to assume that the hypervariable region encompassing the tripeptide marker might represent an antigenically distinct epitope or epitopes, since it is known to be surface orientated. Synthetic peptides representing this region might provide a rapid and simple diagnostic probe for flavivirus antibody responses in infected humans.

6.1.4. Refining the Analysis of Variation Amongst LI Viruses

To assess the extent of genetic variation between very closely related viruses, a rapid and simple sequencing technique was developed which involved sequencing of the virus without cloning the cDNA produced during the PCR reaction. The decision was taken not to plaque purify individual viruses since this could bias the results obtained and possibly select tissue culture adapted variants. Accordingly, the sequence data obtained should represent the consensus sequence for the viral quasispecies present in the original sample. However, variation in the sequence of the quasispecies population was only detected in the IRE4 isolate. The lack of sequence heterogeneity of the quasispecies present in all the other virus samples is not surprising when the high degree of sequence similarity between viruses collected from the same area many years apart is taken into account, e.g. Devon and Thurso viruses.

One of the major questions to be answered was whether or not sequencing a short region of the E gene would provide sufficient information for the construction of a robust phylogeny. Therefore the region of the E gene that encodes two of the genetic markers, i.e. the tripeptide and the hexapeptide markers, was selected for analysis since it is known to contain both conserved and variable regions and can be used to provide very precise flavivirus diagnosis. The viral RNA extracted from crude mouse brain suspension was reverse transcribed and amplified as cDNA by RT-PCR.

Direct solid-phase sequencing was then carried out on the product to determine the nucleotide and deduced amino acid sequences. The phylogenetic analysis of these data was compared with and also combined with the complete E gene sequence data of additional LI viruses obtained by conventional plaque purification and DNA cloning methods.

Despite the very low level of genetic variation detected in the RGFs it still proved possible to construct a tree which reflected the results seen previously in trees constructed using complete E gene sequence data. However, evolution among the geographically isolated microcommunities that were identified in this study could only be analyzed reliably using complete E gene sequence data. Nevertheless, some interesting observations were made. The rapid sequencing method identified a mixed population in the IRE4 isolate. Unfortunately, constraints of time did not permit experiments to be carried out to separate the two virus populations by plaque purification but it is clear from the mixed sequence data that the IRE4 isolate represented a mixture of typical Irish LI virus and typical British LI virus.

6.1.5. Dispersal Patterns & Proposed Evolution of LI Virus.

Phylogenetic analysis of the tick-borne flaviviruses identified a clinal distribution across Asia and Europe (Zanotto *et al.*, 1995). However, the evolution of individual species of flaviviruses within the cline had not been extensively investigated prior to this study. The clinal distribution was identified by the positive correlation between the geographic and genetic distance in the TBE virus serocomplex. However, a similar correlation was not identified within the geographic distribution of LI virus. A random, multidirectional dispersal pattern was identified, implicating the involvement of different dispersal mechanisms from those observed in Europe and Asia.

The predicted temporal and spatial dynamics of LI virus dispersal differ from the classical view of its evolution. It is clear from the phylogenetic analysis of data generated in this study that LI virus emerged in Ireland, less than 600 years ago, and

was later introduced into Scotland, via Wales. It appears that LI virus did not emerge from the forests of Scotland, where it was believed to have existed since the last ice-age, but was introduced directly into the sheep-tick cycle on the moors. However, the classical view and that formulated in this study do agree that LI virus evolution and dispersal have been accelerated as a direct result of the introduction of the sheep industry onto the moorland habitat. The estimated times for the major divergence of virus lineages correlate well with the introduction of sheep onto the Highlands of Scotland and the exportation of sheep to Norway. However, the involvement of migratory birds and terrestrial animals, both wild and domestic, cannot be discounted. Nevertheless, it appears that man, who has been attempting to control the virus since its identification, was largely responsible for the distribution of LI virus in the British Isles.

6.2. Future Work

This study has provided definitive answers to several questions about LI virus. However, many more remain to be resolved. The analysis of LI virus variation carried out in this study has opened additional areas for investigation. The following areas are of particular interest.

1. The interrelationship between the British and Irish viruses in Ireland should be investigated with epizootiological studies. The results reported here imply that the two virus populations inhabit the same tick population. Therefore, one could ask if the viruses are in competition with each other and whether or not this explains the increased virulence characteristics of the British virus collected in Ireland.
2. A greater understanding of the spatial distribution of LI virus in Ireland may help to confirm and explain the introduction and evolution of the virus in Ireland.
3. Only one virus from Wales and the Borders of Scotland was available in the reported studies. Analysis of additional viruses from these locations is required to confirm the dispersal pattern of LI virus evolution that has been proposed in this

study and to ensure that the investigated viruses represent the entire virus population found in these areas.

4. The epizootiological importance of escape variants should be further investigated and the selection pressures involved identified. Type-I and type-II escape variants appear to exist in different geographic areas. The reasons behind this should be studied further. Moreover, it would be interesting to see if type-I or type-II escape variants are selected in the laboratory in the presence of MAb 7.1.

5. An infectious clone of LI virus has recently been developed (Gritsun pers. com.). Site-directed mutagenesis of the infectious clone should pinpoint amino acids important in determining the virulence characteristics of LI virus. The proposed involvement of residues 308, 311, 346 and 356 of the E protein in the virulence characteristics of the escape variants could be conclusively determined. This analysis, which could be carried out in the natural host, the sheep, may also provide insights into the virulence characteristics of the genus *Flavivirus* as a whole.

6. Once the virulence determinants of LI virus have been identified, the infectious clone could be appropriately modified to produce a safe and effective live attenuated vaccine against LI virus. If this aim could be achieved, a similar strategy could be applied to other viruses in the genus *Flavivirus* with a view to developing safe effective vaccines against all pathogenic flaviviruses.

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APPENDICES

Chemical	PK/BA media (ml)
Earle's (basic salt solution) 1 x concentration	150
Lactalbumin hydrolysate 5.0% w/v	20
Bovine serum albumin 0.5% w/v	20
Antibiotic mixture	4
Glucose 10% w/v	2
folic acid (0.01%) and biotine (0.01%)	2
yeast extract 10%	1
sodium bicarbonate 8% solution	4

Appendix 2.1. Formula for PK/BA medium used during plaque assays.

Chemical	CMC overlay (ml)
carboxy methyl cellulose 2.3% w/v	320
Earle's (basic salt solution) 10 x concentration	43
Lactalbumin hydrolysate 5.0% w/v	50
Newborn calf serum	15
Glucose 10% w/v	5
Antibiotic mixture	25
Folic acid (0.01%) and biotine (0.01%)	5
Yeast extract 10%	2.5
mycostatin	10
polymixin	5
sodium bicarbonate 8% solution	30

Appendix 2.2. Formula for CMC overlay used during plaque assays.

```
                525                                                        584
LI-31           CUUAGGGGAA UAUGGGGACG UCUCACUGCU AUGUAGAGUA GCCAGUGGCG UCGACUUGGC
SB-526          .......... .......... .......... .......... .......... ..........
PEN4            .......... .....A.... .......... ......G... .......... ..........
PEN5            .......... .....A.... .......... ......G... .......... ..........
PEN1            .......... .....A.... .......... ......G... .....C.... ..........
PEN2            .......... .....A.... .......... ......G... .....C.... ..........
PEN3            .......... .....A.... .......... ......G... .....C.... ..........
PEN6            .C........ .....A.... .......... ......G... .....C.... ..........
LI-369          .C........ .....A.... .......... ......G... .......... ..........
OBAN1           .C........ .....A.... .......... ......G... .......... ..........
IRE3            .C........ .....A.... .......... ...C..G... .......... ..........
LI-917          .......... .....A.... .....A.... G.....G... .......... ..........
PRES1           .......... .....A.... .......... ......G... .......... ..........
INV13           .......... .....A.... .......... ......G... .......... ..........
INV15           .......... .U...A.... .......... ......G... .......... ..........
THK1            .......... .....A.... .......... ......G... .......... ..........
WEST1           .......... .....A.... .......... ......G... .......... ..........
INV10           .......... .....A.... .......... ......G... .......... ..........
INV4            .......... .....A.... .......... ......G... .......... ..........
INV6            .......... .....A.... .......... ......G... .......... ..........
INV7            ...G...... .....A.... .......... ......G... .......... ..........
INV8            ...G...... .....A.... .......... ......G... .......... ..........
INV9            .......... .....A.... .......... ...C..G... .......... ..........
LOCH4           .......... .....A.... .......... ...C..G... .......... ..........
LI-K            .......... .....A.... .......... ...C..G... .......... ..........
LOCH2           .......... .....A.... .......... ...C..G... .......... ..........
LOCH3           .......... .....A.... .......... ...C..G... .......... ..........
LOCH5           .......... .....A.... .......... ...C..G... .......... ..........
LOCH1           A......... .....A.... .......... ...C..G... .......... ..........
LOCH6           .......... .....A.... .......... ......G... .......... ..........
BRIS1           .......... .....A.... .......... .......... .......... ..........
DEV1            .......... .....A.... .......... .......... .......... ..........
DEV4            .......... .....A.... .......... .......... .......... ..........
LI-A            .......... .....A.... .......... .......... .......... ..........
DEV2            ......A... .....A.... .......... .......... .......... ..........
DEV3            ......A... .....A.... .......... .......... .......... ..........
INV11           .......... .....A.... .......... ......G... .......... ..........
INV12           .......... .....A.... .......... ......G... .......... ..........
INV2            .......... .....A.... .......... ......G... .......... ..........
INV3            .......... .....A.... .......... ......G... .......... ..........
THO1            .......... .....A.... .......... ......G... .......... ..........
THO2            .......... .....A.... .......... ......G... .......... ..........
INV5            .......... .....A.... .......... ......G... ..U....... ..........
HUR1            .......... .....A.... .......... ......G... .......... ..........
INV1            .......... .....A.... .......... ......G... .......... ..........
LI-261          .C........ .....A.... .......... ......G... .......... ..........
INV14           .......... .....A.... .......... ......G... .......... .......A..
LI-NOR          .......... .....A.... ....G..... G.....G... .......... .......A..
LI-G            .C........ .....A.... .......... ......G... .......... ..........
IRE1            .C.......U .....A..U. ....G..... G.....G..C .......... .U........
MA54            .C.......U .....A..U. ....G..... G..C..G... .......... .U........
IRE2            .C.......U .....A..U. ....G..... G..C..G..C .......... .U........
LI-I            .C........ .....A..U. .....U.... G..C..G..U .......... .U...C....
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Appendix 4.1. Multiple alignment of the nucleotide sequences of LI viruses across the RGF, which encompasses nucleotides 525-846.

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                585                                                       644
LI-31           UCAGACCAUC AUCCUGGAGC UUGACAAGAC AGCGGAGCAC CUCCCAACAG CUUGGCAGGU
SB-526          .......... .......... .......... G......... .......... ..........
PEN4            .......... .......... .C........ .......... ..U....... .......A..
PEN5            .......... .......... .C........ .......... ..U....... .......A..
PEN1            .......... .......... .C........ .......... ..U....... .......A..
PEN2            .......... .......... .C........ .......... ..U....... .......A..
PEN3            .......... .......... .C........ .......... ..U....... .......A..
PEN6            .......... .......... .C........ .......... ..U....... .......A..
LI-369          .......... .......... .C........ .......... ..U....... ..........
OBAN1           .......... .......... .C........ .......... ..U....... ..........
IRE3            .......... .......... .......... .......... ..U....... ..........
LI-917          .......... .......... .C........ .......... ..U....... ..........
PRES1           .......... .......... .C........ .......... ..U....... ..........
INV13           C......... .......... .......... .......... ..U....... ..........
INV15           C......... .......... .......... .......... ..U....... ..........
THK1            .......... .......... .C........ ...A...... ..U....... ..........
WEST1           .......... .......... .C........ ...A...... ..U....... ..........
INV10           .......G.. .......... .C........ .......... ..U....... ..........
INV4            .......G.. .......... .C........ .......... ..U....... ..........
INV6            .......... .......... .C........ .......... ..U....... ..........
INV7            .......... .......... .C........ .......... ..U....... ..........
INV8            .......... .......... .C........ .......... ..U....... ..........
INV9            .......... .......... .C........ .......... ..U....... ..........
LOCH4           .......... .......... .C........ .......... ..U....... ..........
LI-K            .......... .......... .C........ .......... ..U....... ..........
LOCH2           .......... .......... .C........ .......... ..U....... ..........
LOCH3           .......... .......... .C........ .......... ..U....... ..........
LOCH5           .......... .......... .C........ .......... ..U....... ..........
LOCH1           .......... .......... .C........ .......... ..U....... ..........
LOCH6           .......... .......... .C........ .......... ..U....... ..........
BRIS1           .......... ...U...... .C........ .......... ..U....... ..........
DEV1            .......... ...U...... .C........ .......... ..U....... ..........
DEV4            .......... ...U...... .C........ .......... ..U....... ..........
LI-A            .......... ...U...... .C........ .......... ..U....... ..........
DEV2            .......... ...U...... .C........ .......... ..U....... ..........
DEV3            .......... ...U...... .C........ .......... ..U....... ..........
INV11           .......... .......... .C........ .......... ..U....... ..........
INV12           .......... .......... .C........ .......... ..U....... ..........
INV2            .......... .......... .C........ .......... ..U....... ..........
INV3            .......... .......... .C........ .......... ..U....... ..........
THO1            .......... .......... .C........ .......... ..U....... ..........
THO2            .......... .......... .C........ .......... ..U....... ..........
INV5            .......... .......... .C........ .......... ..U....... ..........
HUR1            .......... .......... .A........ .......... ..U....... ..........
INV1            .......... .......... .C........ .......... ..U....... ..........
LI-261          .......... .......... .C........ .......... ..U....... ..........
INV14           C......... .......... .C........ .........U ..U....... .C........
LI-NOR          .......... .......... .C........ ...A...... ..U....... .C........
LI-G            .......... .......... .C........ .......... ..U....... ......G...
IRE1            ......UG.. ..U....... ....U..... ......A... ........G. ..........
MA54            ......UG.. ..U....... ....U..... ......A... ........G. ..........
IRE2            ......UG.. ..U....... ....U..... ......A... ........G. ..........
LI-I            .......... ..U....... .G........ G.....A... ........U. ..........
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                645                                                                 704
LI-31           CCACCGAGAU UGGUUUAAUG ACCUGGCUCU GCCGUGGAAG CAUGAGGGGA ACCCACACUG
SB-526          .........C .....C.... .......... .......... .......... ..........
PEN4            .........C .......... .......... A......... .....U..A. .....U.U..
PEN5            .........C .......... .......... A......... .....U..A. .....U.U..
PEN1            .........C .......... .......... A......... .....U..A. .....U....
PEN2            .........C .......... .......... A......... .....U..A. .....U....
PEN3            .........C .......... .......... A......... .....U..A. ..........
PEN6            .........C .......... .......... A......... .....U..A. .....U....
LI-369          .........C .......... .......... C......... .....U..A. .......U..
OBAN1           .........C .......... .......C.. C......... .....U..A. ..........
IRE3            .........C .......... .......... C......... .....U..A. ..........
LI-917          .........C ........C. .......... C......... .....U..A. ..........
PRES1           .........C ......G.C. .......... C..U...... .....U..A. ..........
INV13           .........C .......... .......C.. C......... .....U..A. ..........
INV15           .........C .......... .......C.. C......... .....U..A. ..........
THK1            .........C .......... .......... C......... .....U..A. .....U....
WEST1           .........C .......... .......... C......... .....U..A. .....U....
INV10           .........C .......... .......... C......... .....U..A. ..........
INV4            .........C .......... .......... C......... .....U..A. ..........
INV6            .........C .......... .U........ C......... .....U..A. .....U....
INV7            .........C .......... .......... C......... .....U..A. ..........
INV8            .........C .......... .......... C......... .....U..A. ..........
INV9            .........C .......... .......... C......... .....U..A. .......U..
LOCH4           .........C .......... .......... C......... .....U..A. .......U..
LI-K            .........C .......... .......... C......... .....U..A. .......U..
LOCH2           .........C .......... .......... C......... .....U..A. .......U..
LOCH3           .........C .......... .......... C......... .....U..A. .......U..
LOCH5           .........C .......... .......... C......... .....U..A. .......U..
LOCH1           .........C .......... .......... C......... .....U..A. .......U..
LOCH6           .........C .......... .......... C......... .....U..A. .......U..
BRIS1           .........C .......... .......... C......... .....U..A. ..........
DEV1            .........C .......... .......... C......... .....U..A. ..........
DEV4            .........C .......... .......... C......... .....U..A. ..........
LI-A            .........C .......... .......... C......... .....U..A. ..........
DEV2            .........C .......... .......... C......... .....U..A. ..........
DEV3            .........C .......... .......... C......... .....U..A. ..........
INV11           ...U.....C .......... .......... C......... .....U..A. ..........
INV12           ...U.....C .......... .......... C......... .....U..A. ..........
INV2            .........C .......... .......... C......... .....U..A. .....U....
INV3            .........C .......... .......... C......... .....U..A. .....U....
THO1            .........C .......... .......... C......... .....U..A. ..........
THO2            .........C .......... .......... C......... .....U..A. ..........
INV5            .........C .......... .......... C......... .....U..A. ..........
HUR1            .........C .......... .......... C......... .....U..A. ..........
INV1            .........C .......... .......... C......... .....U..A. .U........
LI-261          .........C .......... .......... C......... .....U..A. ..........
INV14           .........C .....C.... .......... C..A...... .....U..C. ..........
LI-NOR          A..U.....C .......... .......... C......... .....U..A. ..........
LI-G            .........C .....C.... .......... C..A...... .....U..A. ..........
IRE1            .......... .....C.... .U.....C.. ...A...... .....U...G G.....GU..
MA54            .......... .....C.... .U.....C.. ...A...... .....U...G G.....GU..
IRE2            .......... .....C.... .UU....C.. ...A...... .....U...G G.....GU..
LI-I            ...U...... .....C.... .U........ ...A...... ....UU.... .......U..
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                705                                                       764
LI-31           GAACAACGUA GAGAGAUUGG UUGAGUUUGG UGCCCCUCAU GCUGUCAAGA UGGAUGUGUA
SB-526          .........C ..........  ..........  ..........  ..........  ..........
PEN4            ......U.C. ..........  ..........  ..........  ..........  ..........
PEN5            ......U.C. ..........  ..........  ..........  ..........  ..........
PEN1            ......U.C. ..........  ..........  ...U......  ..........  ..........
PEN2            ......U.C. ..........  ..........  ...U......  ..........  ..........
PEN3            ......U.C. ..........  ..........  ...U......  ..........  ..........
PEN6            ......U.C. ..........  ..........  ...U......  ..........  ..........
LI-369          ......U.C. ......C...  ..........  C..U......  ..........  ..........
OBAN1           ......U.C. ......C...  ..........  C..U......  ..........  ..........
IRE3            ......U.C. ......C...  ..........  C.........  ..........  ..........
LI-917          ......U.C. ..........  ..........  ..........C.  ..........  ..........
PRES1           ......U.C. ..........  ..........  ..........  ..........  ..........
INV13           ......U.C. ..........  ..........  ..........  ..........  ..........
INV15           ......U.C. ..........  ..........  ..........  ..........  ..........
THK1            ......U.C. ..........  ..........  ..........  ..........  ..........
WEST1           ......U.C. ..........  ..........  ..........  ..........  ..........
INV10           ......U.C. ......C...  ..........  ..........  ..........  ..........
INV4            ......U.C. ......C...  ..........  ..........  ..........  ..........
INV6            ......U.C. ......C...  ..........  ..........  ..........  ..........
INV7            ......U.C. ..........  ..........  ..........  ..........  ..........
INV8            ......U.C. ..........  ..........  ..........  ..........  ..........
INV9            ......U.C. ..........  ..........  ..........  ..........  ..........
LOCH4           ......U.C. ..........  ..........  ..........  ..........  ..........
LI-K            ......U.C. ..........  ..........  ..........  ..........  ..........
LOCH2           ......U.C. ..........  ..........  ..........  ..........  ..........
LOCH3           ......U.C. ..........  ..........  ..........  ..........  ..........
LOCH5           ......U.C. ..........  ..........  ..........  ..........  ..........
LOCH1           ......U.C. ..........  ..........  ..........  ..........  ..........
LOCH6           ......U.C. ..........  ..........  ......C...  ..........  ..........
BRIS1           ......U.C. ..........  ..........  ..........  ..........  ..........
DEV1            ......U.C. ..........  ..........  ..........  ..........  ..........
DEV4            ......U.C. ..........  ..........  ..........  ..........  ..........
LI-A            ......U.C. ..........  ..........  ..........  ..........  ..........
DEV2            ......U.C. ..........  ..........  ..........  ..........  ..........
DEV3            ......U.C. ..........  ..........  ..........  ..........  ..........
INV11           ......U.C. ..........  ..........  ..........  ..........  ..........
INV12           ......U.C. ..........  ..........  ..........  ..........  ..........
INV2            ......U.C. ..........  ..........  ..........  ..........  ..........
INV3            ......U.C. ..........  ..........  ..........  ..........  ..........
THO1            ......U.C. ..........  ..........  ..........  ..........  ..........
THO2            ......U.C. ..........  ..........  ..........  ..........  ..........
INV5            ......U.C. ..........  ..........  ..........  ..........  ..........
HUR1            ......U.C. ..........  ..........  ...U......  ..........  ..........
INV1            ......U.C. ..........  ..........  ..........  ..........  ..........
LI-261          ......U.CC ..........  ..........  C.........  ..........  ..........
INV14           ......U.C. ..........  ..........  ..........  ..........  ..........
LI-NOR          .........C ..........  ..........  ..........  ..........  ..........
LI-G            ......U.C. ......C...  ..........  C.........  ..........  .......A..
IRE1            .........C ..........  ....A..C..  G.........  ..........  ..........
MA54            .........C ..........  ....A..C..  G.........  ..........  ..........
IRE2            .........C ..........  ....A..C..  G..U......  ..........  ..........
LI-I            .........C ..........  .C..A.....  C..U..C..C  .......G..  ..........
```

	765					824
LI-31	CAACCUUGGA	GAUCAGACUG	GAGUGCUGCU	GAAGGCGCUC	GCAGGGGUUC	CUGUGGCACA
SB-526	....	....	....	....	....	....U.....
PEN4	....	..C.......	....	....	....	....
PEN5	....	..C.......	....	....	....	....
PEN1	....	..C.......	....	....	....	....
PEN2	....	..C.......	....	....	....	....
PEN3	....	..C.......	....	....	....	....
PEN6	....	..C.......	....	....	....	....
LI-369	....	....	....	..G...A..A	....	....
OBAN1	....	....	....	.........A	....	....
IRE3	....	....	....	..G......A	........C.	....
LI-917	....	.....A....	....	....	....	....
PRES1	....	.....A....	....	....	....	....
INV13	....	....	....	.........G	....	....
INV15	....	....	....	.........A	....	....
THK1	....	........A.	....	....	....	....
WEST1	....	........A.	....	....	....	....
INV10	....	..C.......	....	.........A	....	....
INV4	....	..C.......	....	.........A	....	....
INV6	....	....	....	.........A	....	....
INV7	....	....	....	..G....U.A	....	....
INV8	....	....	....	..G....U.A	....	....
INV9	....	....	....	..G......A	....	....
LOCH4	....	....	....	..G......A	....	....
LI-K	....	....	....	.........A	....	....
LOCH2	....	....	....	.........A	....	....
LOCH3	....	....	....	.........A	....	....
LOCH5	....	....	....	.........A	....	....
LOCH1	....	....	....	.........A	....	....
LOCH6	....	....	....	.........A	....	....
BRIS1	....	....	....	.........A	....	....
DEV1	....	....	....	.........A	....	....
DEV4	....	....	....	.........A	....	....
LI-A	....	....	....	.........A	....	....
DEV2	....	....	....	.........A	....	....
DEV3	....	....	....	.........A	....	....
INV11	....	....	....	.........A	....	....
INV12	....	....	....	.........A	....	....
INV2	....	....	....	.........A	....	....
INV3	....	....	....	.........A	....	....
THO1	....	....	....	.........A	....	....
THO2	....	....	....	.........A	....	....
INV5	....	....	....	.........A	....	....
HUR1	....	....	....	.........A	....	....
INV1	....	....	....	.........G	....	....
LI-261	....	....	....	A.........	....	....
INV14	....	....	....	..G......A	....	....
LI-NOR	....	....	....	....	....	....
LI-G	...U......	....	....	.........A	....	....
IRE1	....	....	....	....	....	....A..G..
MA54	....	....	....	....	....	.......G..
IRE2	U.........	....	....	....	....	....A..G..
LI-I	....	....	....	.........G	....	....A..G..

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                825                   846
LI-31           CAUUGAGGGA AACAAGUAUC AC
SB-526          .......... .......... ..
PEN4            ......A... .......... ..
PEN5            ......A... .......... ..
PEN1            .......... .......... ..
PEN2            .......... .......... ..
PEN3            .......... .......... ..
PEN6            .......... .......... ..
LI-369          .......... .......... ..
OBAN1           .......... ......C... ..
IRE3            .......... .......... ..
LI-917          .......... .......... ..
PRES1           .......... .......... ..
INV13           .......... .......... ..
INV15           .......... .......... ..
THK1            .......... .......... ..
WEST1           .......... .......... ..
INV10           .......... .......... ..
INV4            .......... .......... ..
INV6            .......... .......... ..
INV7            .......... .......... ..
INV8            .......... .......... ..
INV9            .......... .......... ..
LOCH4           .......... .......... ..
LI-K            .......... .......... ..
LOCH2           .......... .......... ..
LOCH3           .......... .......... ..
LOCH5           .......... .......... ..
LOCH1           .......... .......... ..
LOCH6           .......... ........C. ..
BRIS1           .......... .......... ..
DEV1            .......... .......... ..
DEV4            .......... .......... ..
LI-A            .......... .......... ..
DEV2            .......... .......... ..
DEV3            .......... .......... ..
INV11           .......... .......... ..
INV12           .......... .......... .U
INV2            .......... .......... ..
INV3            .......... .......... ..
THO1            .......... .......... ..
THO2            .......... .......... ..
INV5            .......... .......... ..
HUR1            .......... .......... ..
INV1            .......... .......... ..
LI-261          .......... .......... ..
INV14           .......... .......... ..
LI-NOR          .......... .......... ..
LI-G            .........G ........C. ..
IRE1            .......... .......... ..
MA54            .......... .......... ..
IRE2            .......... .......... ..
LI-I            .......... .......... ..
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                176                                                 225
LI-31           LGEYGDVSLL CRVASGVDLA QTIILELDKT AEHLPTAWQV HRDWFNDLAL
SB-526          .......... .......... .......... .......... ..........
PEN4            .......... .......... .......... .......... ..........
PEN5            .......... .......... .......... .......... ..........
PEN1            .......... .......... .......... .......... ..........
PEN2            .......... .......... .......... .......... ..........
PEN3            .......... .......... .......... .......... ..........
PEN6            .......... .......... .......... .......... ..........
LI-369          .......... .......... .......... .......... ..........
OBAN1           .......... .......... .......... .......... ..........
IRE3            .......... .......... .......... .......... ..........
LI-917          ........M. .......... .......... .......... ..........
PRES1           .......... .......... .......... .......... .....D....
INV13           .......... .......... .......... .......... ..........
INV15           ...F...... .......... .......... .......... ..........
THK1            .......... .......... .......... .......... ..........
WEST1           .......... .......... .......... .......... ..........
INV10           .......... .......... ..V....... .......... ..........
INV4            .......... .......... ..V....... .......... ..........
INV6            .......... .......... .......... .......... ..........
INV7            .......... .......... .......... .......... ..........
INV8            .......... .......... .......... .......... ..........
INV9            .......... .......... .......... .......... ..........
LOCH4           .......... .......... .......... .......... ..........
LI-K            .......... .......... .......... .......... ..........
LOCH2           .......... .......... .......... .......... ..........
LOCH3           .......... .......... .......... .......... ..........
LOCH5           .......... .......... .......... .......... ..........
LOCH1           .......... .......... .......... .......... ..........
LOCH6           .......... .......... .......... .......... ..........
BRIS1           .......... .......... .......... .......... ..........
DEV1            .......... .......... .......... .......... ..........
DEV4            .......... .......... .......... .......... ..........
LI-A            .......... .......... .......... .......... ..........
DEV2            .......... .......... .......... .......... ..........
DEV3            .......... .......... .......... .......... ..........
INV11           .......... .......... .......... .......... ..........
INV12           .......... .......... .......... .......... ..........
INV2            .......... .......... .......... .......... ..........
INV3            .......... .......... .......... .......... ..........
THO1            .......... .......... .......... .......... ..........
THO2            .......... .......... .......... .......... ..........
INV5            .......... .......... .......... .......... ..........
Hur1            .......... .......... .......... .......... ..........
INV1            .......... .......... .......... .......... ..........
LI-261          .......... .......... .......... .......... ..........
INV14           .......... .......... .......... .......... ..........
LI-Nor          .......... .......... .......... .......... ..........
LI-G            .......... .......... .......... ........R. ..........
IRE1            .......... .......... ..V....... .......... ..........
MA54            .......... .......... ..V....... .......... ..........
IRE2            .......... .......... ..V....... .......... ..........
LI-I            .......... .......... .......... .......... ..........
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Appendix 4.2. Multiple alignment of the deduced amino acid sequence of LI viruses across the RGF, which includes residues 176-282. The genetic markers have been overlined. The single amino acid code has been used.

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                226                                                       282
LI-31           PWKHEGNPHW NNVERLVEFG APHAVKMDVY NLGDQTGVLL KALAGVPVAH IEGNKYH
SB-526          .......... ..A..... .......... .......... .......... .......
PEN4            ....D...Y. ..A..... .......... .......... .......... .......
PEN5            ....D...Y. ..A..... .......... .......... .......... .......
PEN1            ....D...Y. ..A..... .......... .......... .......... .......
PEN2            ....D...Y. ..A..... .......... .......... .......... .......
PEN3            ....D..... ..A..... .......... .......... .......... .......
PEN6            ....D...Y. ..A..... .......... .......... .......... .......
LI-369          ....D..... ..A..... .......... .......... R......... .......
OBAN1           ....D..... ..A..... .......... .......... .......... .....H.
IRE3            ....D..... ..A..... .......... .......... R......... .......
LI-917          ....D..... ..A..... .......... .......... .......... .......
PRES1           ....D..... ..A..... .......... .......... .......... .......
INV13           ....D..... ..A..... .......... .......... .......... .......
INV15           ....D..... ..A..... .......... .......... .......... .......
THK1            ....D...Y. ..A..... .......... .......... .......... .......
WEST1           ....D...Y. ..A..... .......... .......... .......... .......
INV10           ....D..... ..A..... .......... .......... .......... .......
INV4            ....D..... ..A..... .......... .......... .......... .......
INV6            ....D...Y. ..A..... .......... .......... .......... .......
INV7            ....D..... ..A..... .......... .......... R......... .......
INV8            ....D..... ..A..... .......... .......... R......... .......
INV9            ....D..... ..A..... .......... .......... R......... .......
LOCH4           ....D..... ..A..... .......... .......... .......... .......
LOCH2           ....D..... ..A..... .......... .......... .......... .......
LOCH3           ....D..... ..A..... .......... .......... .......... .......
LOCH5           ....D..... ..A..... .......... .......... .......... .......
LOCH1           ....D..... ..A..... .......... .......... .......... .......
LOCH6           ....D..... ..A..... .......... .......... .......... .......
BRIS1           ....D..... ..A..... .......... .......... .......... .......
DEV1            ....D..... ..A..... .......... .......... .......... .......
DEV4            ....D..... ..A..... .......... .......... .......... .......
LI-A            ....D..... ..A..... .......... .......... .......... .......
DEV2            ....D..... ..A..... .......... .......... .......... .......
DEV3            ....D..... ..A..... .......... .......... .......... .......
INV11           ....D..... ..A..... .......... .......... .......... .......
INV12           ....D..... ..A..... .......... .......... .......... .......
INV2            ....D...Y. ..A..... .......... .......... .......... .......
INV3            ....D...Y. ..A..... .......... .......... .......... .......
THO1            ....D..... ..A..... .......... .......... .......... .......
THO2            ....D..... ..A..... .......... .......... .......... .......
INV5            ....D..... ..A..... .......... .......... .......... .......
Hur1            ....D..... ..A..... .......... .......... .......... .......
INV1            ....D..... ..A..... .......... .......... .......... .......
LI-261          ....D..... ..A..... .......... .......... .......... .......
INV14           ....D..... ..A..... .......... .......... R......... .......
LI-Nor          ....D..... ..A..... .......... .......... .......... .......
LI-G            ....D..... ..A..... .......... .......... .......... .......
IRE1            ....D.G.R. ..A..... .......... .......... .......... .......
MA54            ....D.G.R. ..A..... .......... .......... .......... .......
IRE2            ....D.G.R. ..A..... .......... .......... .......... .......
LI-I            ....V..... ..A..... .....R.... .......... .......... .......
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Appendix 4.3. Multiple alignments of the nucleotide sequences of the LI viruses across the complete E gene. To extend the analysis the alignment includes the sequence of viruses determined in previous studies.

```
                1                                                                                                   100
Li-31           UCACGCUGCA CACAUUUGGA AAACAGGGAC UUUGUCACUG GCACCCAAGG GACUACAAGA GUCACCUUGG UGCUGGAGCU GGGUGGAUGC GUAACCAUAA
SB-526          ..G.....U. .......... .......... .....U.... .......... .......... .......... .......... .......... ..........
PEN3            ..G....... .......... .......... .......... .......G.. .......... .....U.... .......... .......... ..........
PEN6            ..G....... .......... .......... .......... .......... .......... .....U.... .......... .......... ..........
IRE3            ..G....... .......... .......... .......... .......G.. .......... .......... .......... .......... ..........
Li-369          .......... ....C..... .......... .......... .......G.. .......... .......... .......... .......... ..........
DEV4            ..G....... .......... .......... .......... .......G.. .......... .......... .......... .......... ..........
Li-A            ..G....... .......... .......... .......... .......G.. .......... .......... .......... .......... ..........
THO1            ..G....... .......... .......... .......... .......G.. .......... ..U....... .......... .......... ..........
THO2            ..G.....U. .......... .......... .......... .......G.. .......... ..U....... .......... .......... ..........
INV6            ..G....... .......... .......... .......... .......G.. .......... .......... .......... .......... ..........
INV1            ..G....... .......... .......... .......... .......G.. .......... .......... .......... .......... ..........
Li-K            .......... .....C.... .......... .......... .......G.. .......... .......... .......... .......... ..........
INV14           ..G..U.... .......... .......... .......... .......G.. .......... .......... .......... .......... ..........
Li-917          ..G....... .......... .......... .......... .......G.. ......G..G .......... .......... .......... ..........
Li-Nor          .......... .......... .......... .......... .......G.. ......G... .......... .......... .......... ..........
Li-G            ..G....... .......... .......... .......... .......G.. .......... .......... .......... .......... ..G.......
Li-261          .......... .......... .......... .......... .......G.. .......... .......... .......... .......... ..G.......
Li-I            .......... ....C..... G......... .......... .......G.. .......... .......... ........U. .......... ..U.......
MA54            .......... ....C..... G......... .......... .......G.. .........G .......... .......... A........U ..U.......

                101                                                                                                 200
Li-31           CCGCUGAGGG GAAGCCCUCA GUGGAUGUGU GGCUUGACGC CAUCUACCAG GAGAGCCCUG CCAAGACACG CGAGUACUGU CUGCACGCUA AGUUAUCAGA
SB-526          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......A.. .......... A......... .......... .......... .......... .......... .......... U.......C. ....G.....
PEN6            .......A.. .......... A......... .......... .......... .......... .......... .......... U.......C. ....G.....
IRE3            .......... .......... A......... .......... .......... .......... .......... .......... U.......C. ....G.....
Li-369          .......... .......... A......... .......... .......... ..A....... .......... .......... U.......C. ....G.....
DEV4            .......... .......... A......... .......... .......... .......... .......... .......... U.......C. ....G.....
Li-A            .......... .......... A......... .......... .......... .......... .......... .......... U.......C. ....G.....
THO1            .......... .......... A......... .......... .......... .......... .......... .........C U.......C. ....G.....
THO2            .......... .......... A......... .......... .......... .......... .......... .........C U.......C. ....G.....
INV6            .......... .......... A......... .......... .......... .......... .......... .......... U.......C. ....G.....
INV1            .......... .......... A......... .......... .......... .......... .......... U......... U.......C. ....G.....
Li-K            .......... .......... A......... .......... U......... .......... .......... .......... U.......C. ....G.....
INV14           .......... .........G A......... .......... .......... .......... .......... .......... U.......C. ....G.....
Li-917          .......... ......U... .......... .......... .......... .......... .......... .......... U.......C. .A..G.....
Li-Nor          .......... .......... A......... .......... .......... .......... .......... .......... U....U..C. ..........
Li-G            .......... .......... A......... .......... .......... .G........ ....A..... .........C U.A.....C. ....G.....
Li-261          ....C..... .......... A......... .......... .......... ....A..... .......... .......... U.......C. ....G.....
Li-I            .......... ......U... A....C.... .......... .......... .......... .......... .......... ........C. .......G..
MA54            .......... ......U... A.......A. ........U. ...U...... .......... .......... .......... U.......C. ....G..G..
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          201                                                                                                       300
Li-31     AACCAAGGUU GCUGCCAGAU GUCCAACGAU GGGACCAGCC GCCCUGGCUG AAGAACGCCA GAUUGGCACA GUGUGCAAGA GGGACCAGAG UGAUCGAGGG
SB-526    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3      G......... .....U.... .......... .........U .UU...A... .......... .......... .......... .......... .........C
PEN6      G......... .....U.... .......... .........U .UU...A... .......... .......... .......... .......... .........C
IRE3      G......... .....U.... .......... .........U .U........ .......... .......... .......... .......... .........C
Li-369    G......... .....U.... .......... .........U .U....A... .......... .......... .......... .......... .........C
DEV4      G..U...... .......... .......... .........U ......A... .......... .......... .......... .......... .........C
Li-A      G..U...... .......... .......... .........U ......A... .......U.. .......... .......... .......... .........C
THO1      G..U...... .....U.... .......... .........U .U....A... .......... .......... .......... .......... .........C
THO2      G..U...... .....U.... .......... .........U .U....A... .......... .......... .......... .......... .........C
INV6      G......... .....U.... .......... ...G.....U .U....A... .......... .......... .......... .......... .........C
INV1      G..U.....A .....U.... .......... .........U ......A... .......... .......... .......... .......... .........C
Li-K      G..U...... .....U.... .......... .........U .U....A... .......... .......... .......... .......... .........C
INV14     G......... .....U.... .......... .........U .U....A... .......... .......... .......... .......... .........C
Li-917    G......... .......... .......... .........U .U....A... .......... .......... .......... ....U..... .........C
Li-Nor    G......... .......... .......... .........U .U....A... .......... .......... .......... ....U..... .........C
Li-G      G......... .....U.... .......... .........U .U....A... .......... .......... .......... .......... .........C
Li-261    G......... .....U.... .......A.. .........U .U....A... .......... .......... .......... .......... .........C
Li-I      .......... .......... .......... .......... .U....A... ....G.A... .......... .......... .......... .........C
MA54      .......... ..G....... .......... ...G.....U .UU.....G. ......A... .......... .......G.. .......... .........C

          301                                                                                                       400
Li-31     UGGGGCAACC ACUGUGGAUU GUUUGGAAAG GGCAGCAUCG UGGCCUGUGU CAAGGCGGCU UGUGAAGCAA AGAAGAAAGC CACGGGCUAC GUGUACGAUG
SB-526    .......... .......... .......... ........U. .......... .......... .......... .......... .......... ..........
PEN3      ........U. .......G.. .......... ..U.....U. .......C.. ......A... .....G.... .......... ......A... ..........
PEN6      ........U. .......G.. .......... ..U.....U. .......C.. ......A... .....G.... .......... ......A... ..........
IRE3      ........U. .......... .......... ........U. .......C.. .......... .......... .......... ......A... ..........
Li-369    .....A..U. .......U.. .......... ........U. .......C.. .......... .......... .......... U.....A... ..........
DEV4      ........U. .......G.. .......... ........U. .......C.. .......... .......... .......... ......A... ..........
Li-A      ........U. .......... .......... ........U. .......C.. .......... .......... .......... ......A... ..........
THO1      ........U. .......G.. .......... .......... .......C.. .......... .......... .......... ......A... ..........
THO2      ........U. .......G.. .......... .......... .......C.. .......... .......... .......... ......A... ..........
INV6      ........U. .......G.. .......... ........U. .......C.. .......... .......... .......... ......A... ..........
INV1      ........U. .......G.. .......... ........U. .......C.. .......... .......... .......... ......A... ..........
Li-K      ........U. .......G.. .......... ........U. .......C.. .......... .......... .......... ......A... ..........
INV14     .......... .......... .......... ..U.....U. .......C.. .......... ..C....... .......... ......A... ..........
Li-917    .......... .......... .......... ........U. .......C.. .......... .......... .......... ......A..U ..........
Li-Nor    ........U. .......... .......... ........U. .......C.. .........C .......... .......... ......A... ..........
Li-G      .....A..U. .......... .......... ........U. .......C.. .......... .......... .......... ......A... ..........
Li-261    ........U. .......... .......... ........U. .......... U..A...... ..C....... .......... ......A...
Li-I      .......... .......G.. .......... ..U.....U. .......C.. .......... ........G. .......... ..U...A... ..........
MA54      .......... .......... .......... ........U. .......C.. ...A.....C .......... .......... ......A..U ..........
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                401                                                                                                  500
Li-31           CCAACAAAAU AGUGUACACA GUGAAGGUUG AACCACACAC GGGAGACUAU GUUGCCGCCA ACGAGACGCA CAAAGGGAGG AAGACGGCGA CUUUUACGGU
SB-526          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......... ..C....... .......... .......... .......... .......... .......... ...G...... .......... ....C.....
PEN6            .......... ..C....... .......... .......... .......... .......... .......... ...G...... .......... ....C.....
IRE3            .......... .........C .......... .......... .......... .......... .U........ ...G...... .......... ....C.....
Li-369          .......... .......... .......... .......... .......... .......... .U........ ...G...... .......... ....C.....
DEV4            .......... .......... .......... .......... .......... .......... .......... ...G...... ..A....... ....C.....
Li-A            .......... .......... .......... .......... .......... .......... .......... ...G...... ..A....... ....C.....
THO1            .......... .......... .......... .......... .......... .......... .......... ...G...... ..A....... ....C.....
THO2            .......... .......... .......... .......... .......... .......... .......... ...G...... ..A....... ....C.....
INV6            .......... .......... .......... .......... .......... .......... .......... ...G...... ..A....... ....C.....
INV1            .......... .......... .......... .......... .......... .......... .......... ...G...... ..A....... ....C.....
Li-K            .......... .......... .......... .......... .......... .......... .......... ...G...... ..A....... ....C.....
INV14           .......... .......... .......... .......... .......... .......... .......... ...G...C.. ..A....... ....C.....
Li-917          .......... .......... .......... .......... .......... .......... .......... ...G...... .......... ....C.....
Li-Nor          .......... .......... .......... .......... .......... .......... .......... ...G...... .......... ....C.....
Li-G            .......... .......... .......... .......... .......... ........U. .U........ ...G...... .......... ....C.....
Li-261          .......... .......... .......... .......... A......... .......... .U........ ...G.A.... .......... .C..C.....
Li-I            .......... .......... .......... .G........ .......... .......... .......... ...G...... .......... ....C.....
MA54            .......... .......... .......... .G........ A..G..U... .......... .......U.. ..GG...... .......... ....C.....

                501                                                                                                  600
Li-31           UUCUUCAGAG AAGACCAUCU UGACCUUAGG GGAAUAUGGG GACGUCUCAC UGCUAUGUAG AGUAGCCAGU GGCGUCGACU UGGCUCAGAC CAUCAUCCUG
SB-526          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......... .......... .......... .........A .......... .......... G.........C .......... .......... ..........
PEN6            .......... .......... .....C.... .........A .......... .......... G.........C .......... .......... ..........
IRE3            .......... .......... .....C.... .........A .......... .......C.. G......... .......... .......... ..........
Li-369          .......... .......... .....C.... .........A .......... .......... G......... .......... .......... ..........
DEV4            .......... ....G..... .......... .........A .......... .......... .......... .......... .......... .......U..
Li-A            .......... .......... .......... .........A .......... .......... .......... .......... .......... .......U..
THO1            .......... .......... .......... .........A .......... .......... G......... .......... .......... ..........
THO2            .......... .......... .......... .........A .......... .......... G......... .......... .......... ..........
INV6            .......... .......... .......... .........A .......... .......... G......... .......... .......... ..........
INV1            .......... .......... .......... .........A .......... .......... G......... .......... .......... ..........
Li-K            .......... ........U. .......... .........A .......... .......C.. G......... .......... .......... ..........
INV14           .......... .......... .......... .........A .......... .......... G......... .......... .A..C..... ..........
Li-917          .......... .......... .......... .........A .........A ....G..... G......... .......... .......... ..........
Li-Nor          C......... .......... .......... .........A ........G. ....G..... G......... .......... .A........ ..........
Li-G            .......... .......... .....C.... .........A .......... .......... G......... .......... .......... ..........
Li-261          ...C...... .......... .....C.... .........A .......... .......... G......... .......... .......... ..........
Li-I            ...C...... ..A....... .A...C.... .........A ..U......U ....G..C.. G..U...... .....U...C .......... ......U...
MA54            .......... ..A....... .....C.... ...U.....A ..U.....G. ....G..C.. G......... .....U.... .......... UG....U...
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                601                                                                                                  700
Li-31           GAGCUUGACA AGACAGCGGA GCACCUCCCA ACAGCUUGGC AGGUCCACCG AGAUUGGUUU AAUGACCUGG CUCUGCCGUG GAAGCAUGAG GGGAACCCAC
SB-526          .......... ....G..... .......... .......... .......... ...C.....C .......... .......... .......... ..........
PEN3            .....C.... .......... ......U... .......... .A........ ...C...... .......... ....A..... .........U ..A.......
PEN6            .....C.... .......... ......U... .......... .A........ ...C...... .......... ....A..... .........U ..A......U
IRE3            .......... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
Li-369          .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
DEV4            .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
Li-A            .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
THO1            .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
THO2            .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
INV6            .....C.... .......... ......U... .......... .......... ...C...... .....U.... ....C..... .........U ..A......U
INV1            .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A..U....
Li-K            .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
INV14           .....C.... .......... ...U..U... .....C.... .......... ...C.....C .......... ....C..A.. .........U ..C.......
Li-917          .....C.... .......... ......U... .......... .......... ...C...... ..C....... ....C..... .........U ..A.......
Li-Nor          .....C.... .......A.. ......U... .....C.... ....A..U.. ...C...... .......... ....C..... .........U ..A.......
Li-G            .....C.... .......... ......U... .......... G......... ...C.....C .......... ....C..A.. .........U ..A.......
Li-261          .....C.... .......... ......U... .......... .......... ...C...... .......... ....C..... .........U ..A.......
Li-I            .....G.... ....G..... A......... ..U....... .......U.. .........C .....U.... .......A.. .........UU ..........
MA54            ........U. .......... A......... ..G....... .......... .........C .....U.... .C.....A.. .........U ...GG.....

                701                                                                                                  800
Li-31           ACUGGAACAA CGUAGAGAGA UUGGUUGAGU UUGGUGCCCC UCAUGCUGUC AAGAUGGAUG UGUACAACCU UGGAGAUCAG ACUGGAGUGC UGCUGAAGGC
SB-526          .......... ..C....... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......... U.C....... .......... .......U.. .......... .......... .......... ......C... .......... ..........
PEN6            .......... U.C....... .......... .......U.. .......... .......... .......... ......C... .......... ..........
IRE3            .......... U.C....... C......... ....C..... .......... .......... .......... .......... .......... ......G...
Li-369          .U........ U.C....... C......... ....C..U.. .......... .......... .......... .......... .......... ......G...
DEV4            .......... U.C....... .......... .......... .......... .......... .......... .......... .......... ..........
Li-A            .......... U.C....... .......... .......... .......... .......... .......... .......... .......... ..........
THO1            .......... U.C....... .......... .......... .......... .......... .......... .......... .......... ..........
THO2            .......... U.C....... .......... .......... .......... .......... .......... .......... .......... ..........
INV6            .......... U.C....... C......... .......... .......... .......... .......... .......... .......... ..........
INV1            .......... U.C....... .......... .......... .......... .......... .......... .......... .......... ..........
Li-K            .U........ U.C....... .......... .......... .......... .......... .......... .......... .......... ..........
INV14           .......... U.C....... .......... .......... .......... .......... .......... .......... .......... ......G...
Li-917          .......... U.C....... .......... .......... ...C...... .......... .......... .........A .......... ..........
Li-Nor          .......... ..C....... .......... .......... .......... .......... .......... .......... .......... ..........
Li-G            .......... U.C....... C......... ....C..... .......... .......... .A.....U.. .......... .......... ..........
Li-261          .......... U.CC...... .......... ....C..... .......... .......... .......... .......... .......... ....A.....
Li-I            .U........ ..C....... .....C..A. ....C..U.. C..C...... .G........ .......... .......... .......... ..........
MA54            GU........ ..C....... ........A. .C..G..... .......... .......... .......... .......... .......... ..........
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            801                                                                                                  900
Li-31       GCUCGCAGGG GUUCCUGUGG CACACAUUGA GGGAAACAAG UAUCACCUGA AGAGUGGUCA UGUGACCUGC GAGGUGGGAC UGGAAAAAUU AAAGAUGAAA
SB-526      .......... ........U. .......... .......... .......... .......... .......... .......... .......... ..........
PEN3        .......... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
PEN6        .......... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
IRE3        ...A...... ..C....... .......... .......... .......... .......... .......... ..A....... ........C. ..........
Li-369      A..A...... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
DEV4        ...A...... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
Li-A        ...A...... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
THO1        ...A...... .......... .......... .......... .......... .......... .......... ..A....... .......GC. ..........
THO2        ...A...... .......... .......... .......... .......... .......... .......... ..A....... .......GC. ..........
INV6        ...A...... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
INV1        ...G...... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
Li-K        ...A...... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
INV14       ...A...... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
Li-917      .......... .......... .......... .......... .......... .......... .......... ..A....... ........C. ...A......
Li-Nor      .......... .......... .......... .......... .......... .......... .......... ..A....... ........C. ..........
Li-G        ...A...... .......... .......... ...G...... ..C....... .......... .......... ..A....... ........C. ..........
Li-261      .......... .......... .......... .......... .......... .......... .........U ..A....... ........C. ...A......
Li-I        ...G...... ........A. .G........ .......... .......... .......... ..C....... ..A....... ........C. ..........
MA54        .......... .......... .G........ .......... .......... .......C.. .......... ..A....... .......... ..........

            901                                                                                                 1000
Li-31       GGUCUCACGU ACACAAUGUG UGAUAAAUCA AAGUUUGCAU GGAAGAGAAC UCCAACAGAC AGUGGGCAUG ACACAGUGGU CAUGGAAGUC ACAUUUUCCG
SB-526      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3        .......... .......... .......... .......... .......... .......... ..C....... .......... U......... .....C....
PEN6        .......... .......... .......... .......... .......... .......... ..C....... .......... U......... .....C....
IRE3        .......... .......... .......... .......... .......... .......... ..C....... .......... U......... .....C..A.
Li-369      .......... .......... .......... .......... .......... .......... ..C....... .......... U......... .....C....
DEV4        .......... ....C..... ...A...... .......... .......... .......... .......... .......... U......... .....C....
Li-A        .......... ....C..... ...A...... .......... .......... .......... .......... .......... U......... .....C....
THO1        .......... ....C..... .......... .......... .......... .......... .......... .......... U......... .....C....
THO2        .......... ....C..... .......... .......... .......... .......... .......... .......... U......... .....C....
INV6        .......... ....C..... ...A...... .G........ .......... .......... .......... .......... .......... .....C....
INV1        .......... ....C..... ...A...... .......... .......... .......... .......... .......... U.........U .....C....
Li-K        .......... ....C..... .......... .......... .......... .......... .......... .......... U......... .....C....
INV14       .......... ....C..... .......... .......... .......... .......... .......... .......... U.....G... .....C....
Li-917      .......... .......... .......... .G........ .......... .......... ..C....... .......... U......... .....C....
Li-Nor      .......... .......... .......... .......... .......... .......... ..C....... .......... U......... .....C....
Li-G        .......... .......... .......... .......... .......... .......... ..C....... .......... U.....G... .....C....
Li-261      .......... .......... .......... .G........ .......... .......... ..C....... .......... U.....G... .....C....
Li-I        .......... .......... .......... C......... .......... .......... ..C....... .......... U......... .....C..U.
MA54        .......... ......C... .......... .....C.... ........G. .......... ..C....... .......... U.....G... .....C....
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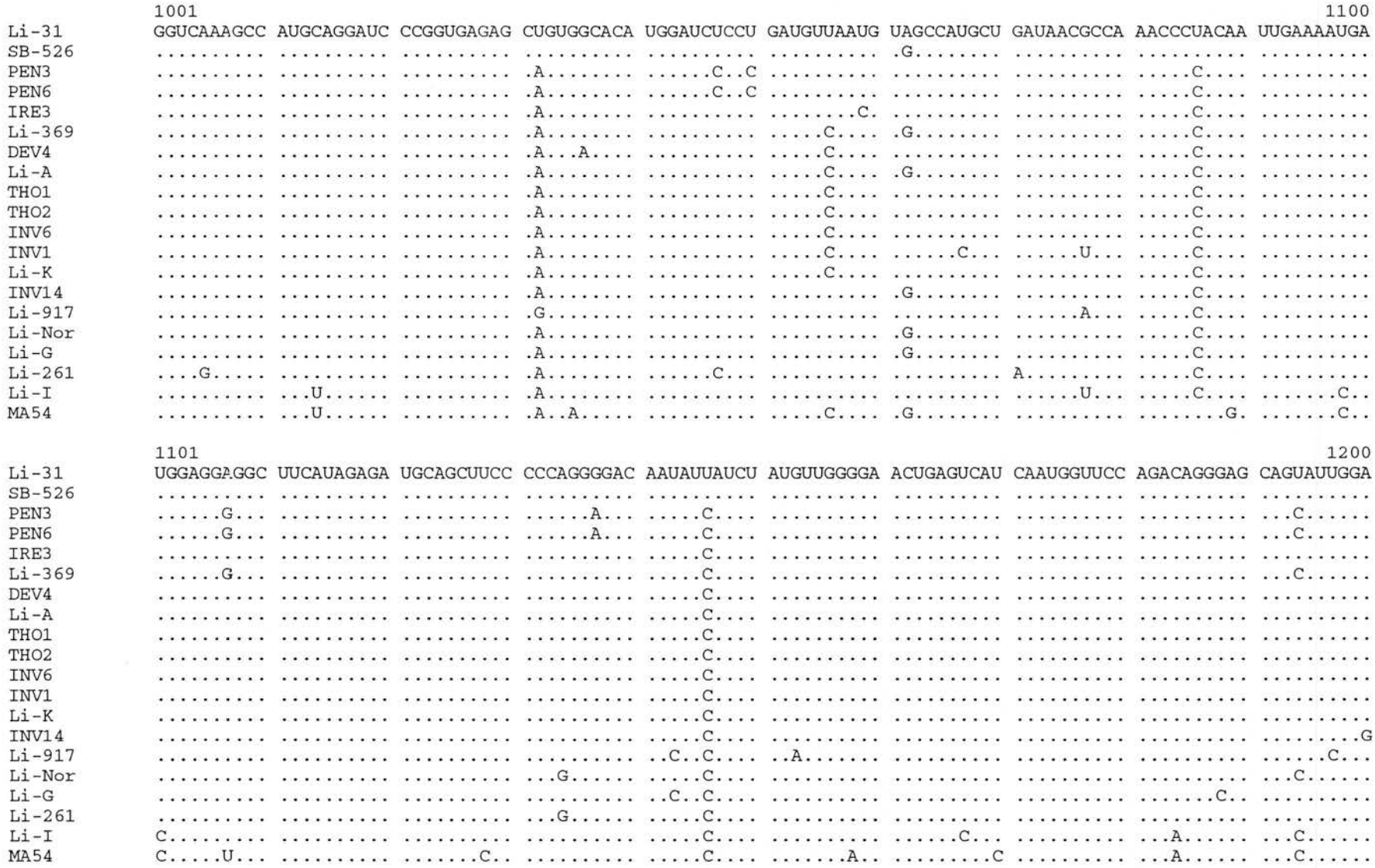
1001 1100
Li-31 GGUCAAAGCC AUGCAGGAUC CCGGUGAGAG CUGUGGCACA UGGAUCUCCU GAUGUUAAUG UAGCCAUGCU GAUAACGCCA AACCCUACAA UUGAAAAUGA
SB-526 .... .G..... ....
PEN3 .... .A..... .....C..C ....C.... .....
PEN6 .... .A..... .....C..C ....C.... .....
IRE3 .... .A..... ....C. ....C.... .....
Li-369 .... .A..... ....C.... .G..... ....C.... .....
DEV4 .... .A...A.... ....C.... ....C.... .....
Li-A .... .A..... ....C.... .G..... ....C.... .....
THO1 .... .A..... ....C.... ....C.... .....
THO2 .... .A..... ....C.... ....C.... .....
INV6 .... .A..... ....C.... ....C.... .....
INV1 .... .A..... ....C.... .....C... .....U... .....C.... .....
Li-K .... .A..... ....C.... ....C.... .....
INV14 .... .A..... .... .G..... ....C.... .....
Li-917 .... .G..... ....A... .....C.... .....
Li-Nor .... .A..... .... .G..... ....C.... .....
Li-G .... .A..... .... .G..... ....C.... .....
Li-261 ....G..... .... .A..... .....C... .... A..... .....C.... .....
Li-I ....U..... ..... .A..... ....U... .....C.... .....C..
MA54 ....U..... ..... .A..A..... ....C.... .G..... ....G. .....C..
1101 1200
Li-31 UGGAGGAGGC UUCAUAGAGA UGCAGCUUCC CCCAGGGGAC AAUAUUAUCU AUGUUGGGGA ACUGAGUCAU CAAUGGUUCC AGACAGGGAG CAGUAUUGGA
SB-526 ....
PEN3 .....G... ....A... .....C.... ....C.....
PEN6 .....G... ....A... .....C.... ....C.....
IRE3 ....C.... ....
Li-369 .....G... ....C.... ....C.....
DEV4 ....C.... ....
Li-A ....C.... ....
THO1 ....C.... ....
THO2 ....C.... ....
INV6 ....C.... ....
INV1 ....C.... ....
Li-K ....C.... ....
INV14 ....C.... ....G
Li-917 ....C..C.... ..A..... ....C...
Li-Nor ....G..... .....C.... ....C.....
Li-G ....C..C.... ....C.. .....
Li-261 ....G..... .....C.... ....
Li-I C..... ....C.... ....C... ....A..... ...C.....
MA54 C.....U... ....C.. ....C.... .....A.. .....C ....A..... ...C.....

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                1201                                                                                                1300
Li-31           AGGGUGUUCC AAACAACCAG GAAAGGUAUA GAAAGACUGA CAGUGAUAGG AGAGCACGCC UGGGACUUUG GAUCCGCUGG UGGCUUCUUC AGCUCAAUUG
SB-526          .......... ...A...... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......... .G........ .......... .........U ....A..... .......... .......... .......... .........U ..........
PEN6            .......... .G........ .......... .........U ....A..... .......... .......... .......... .........U ..........
IRE3            .......... .......... .......... .......... ....A..... .......... .......... .......... .........U ..........
Li-369          .......... .......... .......... .......... ....A..... .......... .......... .......... .........U G.........
DEV4            .......... .......... .......... .......... ....A..... .......... .......... .G........ G........U ..........
Li-A            .......... .......... .......... .......... ....A..... .......... .......... .G........ G........U ..........
THO1            .......... .......... .......... .......... ....A..... .......... .......... .G........ G........U ..........
THO2            .......... .......... .......... .......... ....A..... .......... .......... .G........ G........U ..........
INV6            .......... .......... .......... .......... ....A..... .......... .......... .G........ G........U ..........
INV1            .......... .......... .......... .......... ....A..... .......... .......... .G........ G........  ..........
Li-K            .......... .......... .......... .......... ....A..... .......... .......... .G........ G........U ..........
INV14           .......... .......... .......... .......... ....A..... .......... .......... .......... .........U ..........
Li-917          .......... .......... .......... .......... ....A..... .......... .......... .......... .........U ..........
Li-Nor          .......... .......... .......... .......... ....A..... .......... .......... ....U..... .........U ..........
Li-G            .......... .......... .......... .......... ....A..... .......... .......... .......... .........U ......G...
Li-261          .......... .......... ...G...... ......U... .......... .......... .......... .......... ...U.....U ..........
Li-I            .....U.... .......U.. ......C... .......... .......... ...A..U... .....U.... .......... C........A ......G.C.
MA54            .....C.... .......... ......C... .....G.... .......... ...A..U... .......... ....UAU... C........A G.....G.C.

                1301                                                                                                1400
Li-31           GAAAGGCGGU GCACACGGUU CUUGGUGGUG CUUUCAACAG CAUCUUUGGA GGAGUGGGGU UUCUGCCAAA GCUCUUAAUG GGAGUGGCAC UGGCCUGGCU
SB-526          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
PEN6            .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
IRE3            .......A.. .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
Li-369          .......... .......... .......... .C........ .......... .......... .C........ ....C..... .......... ........U.
DEV4            .......... .......... .......... .C..U..... .......... .......... .......... ....C..... .......... ........U.
Li-A            .......... .......... .......... .C..U..... .......... .......... .......... ....C..... .......... ........U.
THO1            .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
THO2            .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
INV6            .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
INV1            .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
Li-K            .......A.. .......... .......... .C........ .......... ........A. .......... ....C..... .......... ........U.
INV14           .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
Li-917          .......... .......... .......... .C..U..... .......... .......... .......... ....C..... ........U. ........U.
Li-Nor          .......... .......... .......... .C........ .......... .......... .......... ....C..... .......... ........U.
Li-G            .......... .........C .....C.... .C........ .......... .......... .......... ....C..... .......... ........U.
Li-261          .......... .......... .......... .C........ ...U...... .....A.... .......... ....C..... .......... ........U.
Li-I            .......A.. ......A... .......... .C........ U......... .......... .......... A...C..... .......... ....A...U.
MA54            .G..A..... .......... ........C. .C........ UC........ .......... .......... ....C..... .......... ........U.
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          1401                                                                                                1488
Li-31     GGGACUUAAC ACAAGGAACC CCACAAUGUC CAUGAGCUUU CUCUUGACUG GAGGUCUGGU CUUGGCCAUG ACUCUCGGGG UGGGAGCG
SB-526    .......... .......... .......... ...A...... .......... .......... .......... .......... ........
PEN3      ......G... .......... .......... .......... ..U...G... .......... .......... .......... ........
PEN6      ......G... U......... .......... .......... ..U...G... .......... .......... .......... ........
IRE3      ......A... .......... .......... .......... ......G... .......... .......... ..C....... ........
Li-369    ......G... .......... .......... .......... ......G... .......... .......... .......... ........
DEV4      ......G... .......... .......... .......... ...C..G... .......... .......... .......... ........
Li-A      ......G... .......... .......... .......... ...C..G... .......... .......... .......... ........
THO1      ......G... .......... .......... .......... ...C..G... .......... .......... ........A. ........
THO2      ......G... .......... .......... .......... ...C..G... .......... .......... ........A. ........
INV6      ......G... .......... .......... .......... ...C..G... .......... .......... .......... ........
INV1      ......G... .........U. .......... .......... ...C..G... .......... .......... .......... ........
Li-K      ......G... .......... .......... .......... ...C..G.C. .......... .......... .......... ........
INV14     ......G... .......... .......... .......... ...C..G.G. .......... .......... .......... ........
Li-917    ......G... .......... .......... .......... ......G... .......... .......... .......... ........
Li-Nor    ......G... .......... .U........ .......... ...A..G... .......... .......... .......... ........
Li-G      ......G... .......... .......... .......... ..U...G... .......... .......... .......... ........
Li-261    ......G... .......... .......... .......... ......G... .......... .......... .......... ........
Li-I      ....U.G... ..G....... .......... .......... ..U...G... .......... .C........ .......... ........
MA54      ...UU.G... U.G.....U. .U........ .......... ......G... .......... .......... .......... ........
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Appendix 4.4. Multiple alignments of deduced amino acid sequences of LI viruses across the complete E gene. The single amino acid code has been used. Additional sequences of previously determined viruses have been included to extend the analysis.

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                1                                                                                                   100
LI-31           SRCTHLENRD FVTGTQGTTR VTLVLELGGC VTITAEGKPS VDVWLDAIYQ ESPAKTREYC LHAKLSETKV AARCPTMGPA ALAEERQIGT VCKRDQSDRG
SB-526          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
PEN6            .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
INV1            .......... .......... .......... .......... M......... .......... .......... .......... ..T....... ..........
LI-A            .......... .......... .......... .......... M......... .......... .......... .......... ..T....... ..........
DEV4            .......... .......... .......... .......... M......... .......... .......... .......... ..T....... ..........
LI-K            .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
THO1            .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
THO2            .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
LI-Nor          .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
INV14           .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
LI-369          .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
IRE3            .......... .......... .......... .......... M......... .......... .......... .......... V......... ..........
LI-917          .......... .......... .......... .......... .......... .......... .......... .......... V.T....... ..........
INV6            .......... .......... .......... .......... M......... .......... .......... .......... V.T....... ..........
LI-G            .......... .......... .......... .......... M......... G......... .......... .......... V.T....... ..........
LI-261          .......... .......... .......... .......... M......... .N........ .......... .......... V.T....... ..........
LI-I            .......... .......... .......... .......... M......... .......... .......... .......... V.T..H.... ..........
MA54            .......... .......... .......... .......... M.....S... .......... .......... .......... V....H.... ..R.......

                101                                                                                                 200
LI-31           WGNHCGLFGK GSIVACVKAA CEAKKKATGY VYDANKIVYT VKVEPHTGDY VAANETHKGR KTATFTVSSE KTILTLGEYG DVSLLCRVAS GVDLAQTIIL
SB-526          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3            .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........
PEN6            .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........
INV1            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-A            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
DEV4            .......... .......... .......... .......... .......... .......... .......... .S........ .......... ..........
LI-K            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
THO1            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
THO2            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-Nor          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
INV14           .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-369          .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
IRE3            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-917          .......... .......... .......... .......... .......... .......... .......... .......... ...M...... ..........
INV6            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-G            .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-261          .......... .......... .......... .......... .......... ........E. .......... .......... .......... ..........
LI-I            .......... .......... .......M.. .......... .......... .......... .......... .......... .......... ..........
MA54            .......... .......... .......... .......... .......... .......R.. .......... .......D.. .......... .......V..
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                201                                                                                                          300
LI-31     ELDKTAEHLP TAWQVHRDWF NDLALPWKHE GNPHWNNVER LVEFGAPHAV KMDVYNLGDQ TGVLLKALAG VPVAHIEGNK YHLKSGHVTC EVGLEKLKMK
SB-526    .......... .......... .......... .......A.. .......... .......... .......... .......... .......... ..........
PEN3      .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
PEN6      .......... .......... .........D ...Y...A.. .......... .......... .......... .......... .......... ..........
INV1      .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
LI-A      .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
DEV4      .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
LI-K      .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
THO1      .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
THO2      .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
LI-Nor    .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
INV14     .......... .......... .........D .......A.. .......... .......... .....R.... .......... .......... ..........
LI-369    .......... .......... .........D .......A.. .......... .......... .....R.... .......... .......... ..........
IRE3      .......... .......... .........D .......A.. .......... .......... .....R.... .......... .......... ..........
LI-917    .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
INV6      .......... .......... .........D ...Y...A.. .......... .......... .......... .......... .......... ..........
LI-G      .......... ...R...... .........D .......A.. .......... .......... .......... .......... .......... ..........
LI-261    .......... .......... .........D .......A.. .......... .......... .......... .......... .......... ..........
LI-I      .......... .......... .........V .......A.. .......... R......... .......... .......... .......A.. ..........
MA54      .......... .......... .........D .G.R...A.. .......... .......... .......... .......... .......... ..........

                301                                                                                                          400
LI-31     GLTYTMCDKS KFAWKRTPTD SGHDTVVMEV TFSGSKPCRI PVRAVAHGSP DVNVAMLIT? NPTIENDGGG FIEMQLPPGD NIIYVGELSH QWFQTGSSIG
SB-526    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
PEN6      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
INV1      .......E.. .......... .......... .......... .......... .....T.... .......... .......... .......... ..........
LI-A      .......E.. .......... .......... .......... .......... .......... .......... .......... .......... ..........
DEV4      .......E.. .......... .......... .......... .....T.... .......... .......... .......... .......... ..........
LI-K      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
THO1      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
THO2      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-Nor    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
INV14     .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-369    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
IRE3      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-917    .......... R......... .......... .......... .......... .......... .......... .......... ....I..... ..........
INV6      .......E.. R......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-G      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-261    .......... R......... .......... .......... .......... .......... .......... .......... .......... ..........
LI-I      .......... Q......... .......... .......... .......... .......... .......... .......... .......... ....K.....
MA54      .....T.... ......A... .......... .......... .......... .......... .......... .......... .......... ....K.....
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          401                                                                                                  496
LI-31     RVFQTTRKGI ERLTVIGEHA WDFGSAGGFF SSIGKAVHTV LGGAFNSIFG GVGFLPKLLM GVALAWLGLN TRNPTMSMSF LLTGGLVLAM TLGVGA
SB-526    ....K..... .......... .......... .......... .......... .......... .......... .......I.. .......... ......
PEN3      .......... ...S...... .......... .......... .......... .......... .......... .......... ..A....... ......
PEN6      .......... ...S...... .......... .......... .......... .......... .......... S......... ..A....... ......
INV1      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
LI-A      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
DEV4      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
LI-K      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
THO1      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
THO2      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
LI-Nor    .......... .......... .......... .......... .......... .......... .......... .......... .MA....... ......
INV14     .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
LI-369    .......... .......... .......... G......... .......... .......... .......... .......... ..A....... ......
IRE3      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
LI-917    .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
INV6      .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
LI-G      .......... .......... .......... ..V....... .......... .......... .......... .......... ..A....... ......
LI-261    .......... .......... .......... .......... .......... .......... .......... .......... ..A....... ......
LI-I      .......... .......... .........L ..V....... .......... .......... .......... .......... ..A....... ......
MA54      .......... .......... .....I...L G.V....... .......L.. .......... .......... S......... ..A....... ......
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Virus	Area (mm^2)									
DEV1	0.938	1.875	1.938	2.000	2.063	2.125	2.188	2.188	2.188	2.250
DEV2	1.813	2.313	2.500	2.625	2.750	2.875	2.938	3.063	3.313	3.500
DEV3	2.063	2.250	2.500	2.563	2.688	2.875	3.000	3.250	3.688	3.938
DEV4	2.250	2.313	2.813	2.813	3.000	3.063	3.125	3.188	3.188	3.938
INV1	1.000	1.375	1.750	2.000	2.063	2.313	2.375	2.375	2.500	2.500
INV6	2.625	2.750	2.875	2.875	3.000	3.125	3.250	3.375	3.563	3.750
INV14	1.438	2.375	2.750	2.750	2.938	3.000	3.063	3.563	3.688	3.875
IRE2	4.125	4.188	5.500	5.625	5.875	6.000	6.563	6.625	6.875	8.375
IRE3	2.875	2.938	4.813	4.813	5.500	5.938	6.813	7.125	7.250	7.688
LI/A	1.125	2.000	2.813	2.813	2.875	3.000	4.375	4.500	4.563	5.250
LI/369	2.250	2.625	2.938	3.563	4.000	4.000	4.125	4.250	4.438	4.813
LOCH2	2.688	3.563	4.125	4.563	4.625	4.875	5.688	6.938	8.250	8.625
LOCH6	2.000	2.188	2.438	2.750	2.875	3.313	3.563	3.625	4.188	4.563
PEN3	2.063	2.125	2.625	2.625	2.875	3.000	3.188	3.375	3.438	5.188
PEN4	1.063	2.000	2.313	2.438	2.688	2.875	3.000	3.063	3.375	4.250
PEN6	1.125	1.750	1.875	2.125	2.188	2.375	2.438	2.625	3.438	3.625
PRES1	2.500	2.563	2.563	2.688	2.750	2.938	3.000	3.188	3.188	3.313
THO2	3.313	3.563	3.688	4.250	4.500	4.500	4.500	5.125	5.500	6.188
BL80	2.750	2.875	3.375	3.625	3.750	4.125	4.750	4.875	5.438	6.375

Appendix 5.1. Area of 10 plaques (mm^2) produced by LI viruses after 96 hours infection of PK cell monolayers.

Appendix 5.2. Cumulative mortality of all C57/BL mice and that observed in male (M) and female (F) animals during 21 days after subcutaneous inoculation with 10 LI viruses.

		Days Post Inoculation															
Virus	Gender	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
DEV4	M	0	0	0	0	0	3	3	3	5	5	5	5	6	6	6	6
	F	0	0	0	0	1	3	5	5	5	6	7	7	7	7	7	7
	Total	0	0	0	0	1	6	8	8	10	11	12	12	13	13	13	13
INV1	M	0	0	0	1	3	6	8	9	9	9	9	9	9	9	9	9
	F	0	0	1	2	3	6	6	8	9	10	10	10	10	10	10	10
	Total	0	0	1	3	6	12	14	17	18	19	19	19	19	19	19	19
INV6	M	0	0	0	0	2	3	3	3	4	4	4	4	4	4	4	4
	F	0	0	0	3	4	6	9	9	9	9	9	9	9	9	9	9
	Total	0	0	0	3	6	9	12	12	13	13	13	13	13	13	13	13
IRE2	M	0	0	1	4	4	7	7	9	9	9	9	9	9	9	9	9
	F	0	1	5	8	10	10	10	10	10	10	10	10	10	10	10	10
	Total	0	1	6	12	14	17	17	19	19	19	19	19	19	19	19	19
IRE3	M	0	0	0	0	0	2	4	5	6	6	7	7	8	8	8	8
	F	0	0	2	5	8	9	9	10	10	10	10	10	10	10	10	10
	Total	0	0	2	5	8	11	13	15	16	16	17	17	18	18	18	18
LI/A	M	0	0	0	0	0	0	1	1	3	3	3	3	3	3	3	3
	F	0	0	0	0	1	4	4	4	4	4	4	4	4	4	4	4
	Total	0	0	0	0	1	4	5	5	7	7	7	7	7	7	7	7
PEN3	M	0	0	2	3	4	4	6	8	8	8	9	9	9	9	9	9
	F	0	0	0	0	0	1	2	2	4	4	4	4	5	5	5	5
	Total	0	0	2	3	4	5	8	10	12	12	13	13	14	14	14	14
PEN6	M	0	0	0	0	0	0	0	1	2	2	2	2	2	2	2	2
	F	0	0	0	0	1	1	1	2	2	2	2	3	3	3	3	3
	Total	0	0	0	0	1	1	1	3	4	4	4	5	5	5	5	5
PRES1	M	0	0	0	0	0	2	5	5	5	5	5	5	5	6	6	6
	F	0	0	0	1	5	5	7	8	8	8	9	9	9	9	9	9
	Total	0	0	0	1	5	7	12	13	13	13	14	14	14	15	15	15
SB/526	M	0	0	0	1	5	5	6	6	6	7	7	7	7	7	7	7
	F	0	0	0	1	3	5	6	6	7	7	8	8	8	8	8	8
	Total	0	0	0	2	8	10	12	12	13	14	15	15	15	15	15	15

Virus	Mouse Strain	Days Post Inoculation																
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
INV6	C57/BL	0	0	0	0	3	6	9	12	12	13	13	13	13	13	13	13	13
	SW	0	1	2	6	10	13	13	14	15	16	16	16	16	16	16	16	16
IRE2	C57/BL	0	0	1	6	12	14	17	17	19	19	19	19	19	19	19	19	19
	SW	0	11	14	19	20	20	20	20	20	20	20	20	20	20	20	20	20
PEN6	C57/BL	0	0	0	0	0	1	1	1	3	4	4	4	5	5	5	5	5
	SW	0	0	1	2	4	8	10	11	15	15	16	16	16	16	16	16	16

Appendix 5.3. Comparison of the cumulative mortality resulting from the subcutaneous inoculation of three LI viruses in C57/BL and SW mice.

Days post inoculation	Virus									
	DEV4	INV1	INV6	IRE2	IRE3	LI/A	PEN3	PEN6	PRES1	SB/526
Day 1	0.00	0.00	0.00	1.12	2.12	0.00	0.00	1.52	1.30	0.00
	0.00	1.43	0.00	0.00	2.10	1.43	0.82	1.52	0.00	0.00
	0.00	1.52	1.30	1.52	1.52	0.00	0.00	1.12	1.82	0.00
	*	0.00	0.82	2.12	1.52	0.00	1.30	0.00	1.12	0.82
	*	0.00	0.00	1.43	1.73	1.52	1.12	0.00	0.82	1.12
Day 3	0.00	0.00	0.00	1.30	0.00	1.52	0.00	0.00	0.00	0.00
	1.60	1.52	0.00	2.22	0.00	1.52	0.00	0.00	0.00	0.82
	0.00	1.52	1.52	1.60	1.90	0.82	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	*	0.82	0.00	0.00	0.00	0.82	0.00
	0.00	1.43	0.00	0.82	1.67	1.12	0.00	0.00	0.00	0.00
Day 5	0.00	0.00	0.00	0.00	0.82	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.82	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.82	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	2.05	0.00	0.00	0.00	1.67	0.00
Day 7	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix 5.4. Viraemia titres (log_{10} p.f.u./0.2 ml) detected in the blood of C57/BL mice during the first 7 days post inoculation with 10 LI viruses. * indicates that no virus isolation was attempted.

Appendix 5.5. Virus titres (log_{10} p.f.u./0.2 gm) detected in the brains of C57/BL mice during the first 7 days post inoculation. and SW mice on day 6 post inoculation. * indicates that no virus isolation was attempted.

Mouse Strain	Days post inoculation	Virus									
		DEV4	INV1	INV6	IRE2	IRE3	LI/A	PEN3	PEN6	PRES1	SB/526
C57/BL	Day 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Day 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Day 5	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	1.82	*	0.00	0.00	2.60	0.00
		0.00	0.00	0.00	1.82	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Day 7	0.00	0.00	0.00	0.82	0.00	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	*	0.00	0.00	0.00	0.00	3.39	0.00
		0.00	0.00	0.00	1.60	3.19	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	0.00	3.45	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00	*	0.00	0.00	0.00	0.00	0.00	0.00
SW	Day 6			5.08	8.70				1.82		
				8.56	6.43				*		
				4.67	4.48				2.78		
				7.09	8.30				5.10		
				4.05	8.37				7.53		